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(54) Title: TRANSGENIC NON-HUMAN ANIMALS CAPABLE OF PRODUCING HETEROLOGOUS ANTIBODIES

(57) Abstract

The invention relates to transgenic non-human animals capable of producing heterologous antibodies and methods for producing human sequence antibodies which bind to human antigens with substantial affinity.

TRANSGENIC NON-HUMAN ANIMALS CAPABLE OF PRODUCING HETEROLOGOUS ANTIBODIES

TECHNICAL FIELD

The invention relates to transgenic non-human animals capable of producing heterologous antibodies, transgenes used to produce such transgenic animals, transgenes capable of functionally rearranging a heterologous D gene in V-D-J recombination, immortalized B-cells capable of producing heterologous antibodies, methods and transgenes for producing heterologous antibodies of multiple isotypes, methods and transgenes for producing heterologous antibodies wherein a variable region sequence comprises somatic mutation as compared to germline rearranged variable region sequences, transgenic nonhuman animals which produce antibodies having a human primary sequence and which bind to human antigens, hybridomas made from B cells of such transgenic animals, and monclonal antibodies expressed by such hybridomas.

BACKGROUND OF THE INVENTION

One of the major impediments facing the development of in vivo therapeutic and diagnostic applications for monoclonal antibodies in humans is the intrinsic immunogenicity of non-human immunoglobulins. For example, when immunocompetent human patients are administered therapeutic doses of rodent monoclonal antibodies, the patients produce antibodies against the rodent immunoglobulin sequences; these human anti-mouse antibodies (HAMA) neutralize the therapeutic antibodies and can cause acute toxicity. Hence, it is desirable to produce human immunoglobulins that are reactive

with specific human antigens that are promising therapeutic and/or diagnostic targets. However, producing human immunoglobulins that bind specifically with human antigens is problematic.

5   The present technology for generating monoclonal antibodies involves pre-exposing, or priming, an animal (usually a rat or mouse) with antigen, harvesting B-cells from that animal, and generating a library of hybridoma clones. By screening a hybridoma population for antigen binding specificity (idiotype) and also screening for immunoglobulin class (isotype), it is possible to select hybridoma clones that secrete the desired antibody.

However, when present methods for generating monoclonal antibodies are applied for the purpose of generating human antibodies that have binding specificities for human antigens, obtaining B-lymphocytes which produce human immunoglobulins a serious obstacle, since humans will typically not make immune responses against self-antigens.

Hence, present methods of generating human monoclonal antibodies that are specifically reactive with human antigens are clearly insufficient. It is evident that the same limitations on generating monoclonal antibodies to authentic self antigens apply where non-human species are used as the source of B-cells for making the hybridoma.

25   The construction of transgenic animals harboring a functional heterologous immunoglobulin transgene are a method by which antibodies reactive with self antigens may be produced. However, in order to obtain expression of therapeutically useful antibodies, or hybridoma clones producing such antibodies, the transgenic animal must produce transgenic B cells that are capable of maturing through the B lymphocyte development pathway. Such maturation requires the

presence of surface IgM on the transgenic B cells, however
isotypes other than IgM are desired for therapeutic uses.
Thus, there is a need for transgenes and animals harboring
such transgenes that are able to undergo functional V-D-J

5   rearrangement to generate recombinational diversity and
junctional diversity. Further, such transgenes and transgenic
animals preferably include cis-acting sequences that
facilitate isotype switching from a first isotype that is
required for B cell maturation to a subsequent isotype that

10 has superior therapeutic utility.

A number of experiments have reported the use of
transfected cell lines to determine the specific DNA sequences
required for Ig gene rearrangement (reviewed by Lewis and
Gellert (1989), Cell, 59, 585-588). Such reports have

15 identified putative sequences and concluded that the
accessibility of these sequences to the recombinase enzymes
used for rearrangement is modulated by transcription
(Yancopoulos and Alt (1985), Cell, 40, 271-281). The
sequences for V(D)J joining are reportedly a highly conserved,

20 near-palindromic heptamer and a less well conserved AT-rich
nanomer separated by a spacer of either 12 or 23 bp (Tonegawa
(1983), Nature, 302, 575-581; Hesse, et al. (1989), Genes in
Dev., 3, 1053-1061). Efficient recombination reportedly
occurs only between sites containing recombination signal

25 sequences with different length spacer regions.

Ig gene rearrangement, though studied in tissue
culture cells, has not been extensively examined in transgenic
mice. Only a handful of reports have been published
describing rearrangement test constructs introduced into mice

30 [Buchini, et al. (1987), Nature, 326, 409-411 (unrearranged
chicken λ transgene); Goodhart, et al. (1987) , Proc. Natl.
Acad. Sci. USA, 84, 4229-4233) (unrearranged rabbit κ gene);

and Bruggemann, et al. (1989), Proc. Natl. Acad. Sci. USA, 86, 6709-6713 (hybrid mouse-human heavy chain)]. The results of such experiments, however, have been variable, in some cases, producing incomplete or minimal rearrangement of the

5   transgene.

Further, a variety of biological functions of antibody molecules are exerted by the Fc portion of molecules, such as the interaction with mast cells or basophils through Fcε, and binding of complement by Fcμ or Fcγ, it further is

10   desirable to generate a functional diversity of antibodies of a given specificity by variation of isotype.

Although transgenic animals have been generated that incorporate transgenes encoding one or more chains of a heterologous antibody, there have been no reports of

15   heterologous transgenes that undergo successful isotype switching. Transgenic animals that cannot switch isotypes are limited to producing heterologous antibodies of a single isotype, and more specifically are limited to producing an isotype that is essential for B cell maturation, such as IgM

20   and possibly IgD, which may be of limited therapeutic utility. Thus, there is a need for heterologous immunoglobulin transgenes and transgenic animals that are capable of switching from an isotype needed for B cell development to an isotype that has a desired characteristic for therapeutic use.

25   Based on the foregoing, it is clear that a need exists for methods of efficiently producing heterologous antibodies, e.g. antibodies encoded by genetic sequences of a first species that are produced in a second species. More particularly, there is a need in the art for heterologous

30   immunoglobulin transgenes and transgenic animals that are capable of undergoing functional V-D-J gene rearrangement that incorporates all or a portion of a D gene segment which

contributes to recombinational diversity. Further, there is a need in the art for transgenes and transgenic animals that can support V-D-J recombination and isotype switching so that (1) functional B cell development may occur, and (2)

5   therapeutically useful heterologous antibodies may be produced. There is also a need for a source of B cells which can be used to make hybridomas that produce monoclonal antibodies for therapeutic or diagnostic use in the particular species for which they are designed. A heterologous

10   immunoglobulin transgene capable of functional V-D-J recombination and/or capable of isotype switching could fulfill these needs.

In accordance with the foregoing object transgenic nonhuman animals are provided which are capable of producing a

15   heterologous antibody, such as a human antibody.

Further, it is an object to provide B-cells from such transgenic animals which are capable of expressing heterologous antibodies wherein such B-cells are immortalized to provide a source of a monoclonal antibody specific for a

20   particular antigen.

In accordance with this foregoing object, it is a further object of the invention to provide hybridoma cells that are capable of producing such heterologous monoclonal antibodies.

25   Still further, it is an object herein to provide heterologous unrearranged and rearranged immunoglobulin heavy and light chain transgenes useful for producing the aforementioned non-human transgenic animals.

Still further, it is an object herein to provide

30   methods to disrupt endogenous immunoglobulin loci in the transgenic animals.

Still further, it is an object herein to provide
methods to induce heterologous antibody production in the
aforementioned transgenic non-human animal.

A further object of the invention is to provide
5 methods to generate an immunoglobulin variable region gene
segment repertoire that is used to construct one or more
transgenes of the invention.

The references discussed herein are provided solely
for their disclosure prior to the filing date of the present
10 application. Nothing herein is to be construed as an
admission that the inventors are not entitled to antedate such
disclosure by virtue of prior invention.

SUMMARY OF THE INVENTION

15   Transgenic nonhuman animals are provided which are
capable of producing a heterologous antibody, such as a human
antibody. Such heterologous antibodies may be of various
isotypes, including: IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2,
IgA$_{sec}$, IgD, of IgE. In order for such transgenic nonhuman
20 animals to make an immune response, it is necessary for the
transgenic B cells and pre-B cells to produce surface-bound
immunoglobulin, particularly of the IgM (or possibly IgD)
isotype, in order to effectuate B cell development and
antigen-stimulated maturation. Such expression of an IgM (or
25 IgD) surface-bound immunoglobulin is only required during the
antigen-stimulated maturation phase of B cell development, and
mature B cells may produce other isotypes, although only a
single switched isotype may be produced at a time.

Typically, a cell of the B-cell lineage will produce
30 only a single isotype at a time, although cis or trans
alternative RNA splicing, such as occurs naturally with the μ_s
(secreted μ) and μ_M (membrane-bound μ) forms, and the μ and δ

immunoglobulin chains, may lead to the contemporaneous
expression of multiple isotypes by a single cell. Therefore,
in order to produce heterologous antibodies of multiple
isotypes, specifically the therapeutically useful IgG, IgA,
and IgE isotypes, it is necessary that isotype switching
occur. Such isotype switching may be classical class-
switching or may result from one or more non-classical isotype
switching mechanisms.

The invention provides heterologous immunoglobulin
transgenes and transgenic nonhuman animals harboring such
transgenes, wherein the transgenic animal is capable of
producing heterologous antibodies of multiple isotypes by
undergoing isotype switching. Classical isotype switching
occurs by recombination events which involve at least one
switch sequence region in the transgene. Non-classical
isotype switching may occur by, for example, homologous
recombination between human σ_μ and human Σ_μ sequences (δ-
associated deletion). Alternative non-classical switching
mechanisms, such as intertransgene and/or interchromosomal
recombination, among others, may occur and effectuate isotype
switching. Such transgenes and transgenic nonhuman animals
produce a first immunoglobulin isotype that is necessary for
antigen-stimulated B cell maturation and can switch to encode
and produce one or more subsequent heterologous isotypes that
have therapeutic and/or diagnostic utility. Transgenic
nonhuman animals of the invention are thus able to produce, in
one embodiment, IgG, IgA, and/or IgE antibodies that are
encoded by human immunoglobulin genetic sequences and which
also bind specific human antigens with high affinity.

The invention also encompasses B-cells from such
transgenic animals that are capable of expressing heterologous
antibodies of various isotypes, wherein such B-cells are

immortalized to provide a source of a monoclonal antibody specific for a particular antigen. Hybridoma cells that are derived from such B-cells can serve as one source of such heterologous monoclonal antibodies.

5   The invention provides heterologous unrearranged and rearranged immunoglobulin heavy and light chain transgenes capable of undergoing isotype switching in vivo in the aforementioned non-human transgenic animals or in explanted lymphocytes of the B-cell lineage from such transgenic

10   animals. Such isotype switching may occur spontaneously or be induced by treatment of the transgenic animal or explanted B-lineage lymphocytes with agents that promote isotype switching, such as T-cell-derived lymphokines (e.g., IL-4 and IFN$_\gamma$).

15   Still further, the invention includes methods to induce heterologous antibody production in the aforementioned transgenic non-human animal, wherein such antibodies may be of various isotypes. These methods include producing an antigen-stimulated immune response in a transgenic nonhuman animal for

20   the generation of heterologous antibodies, particularly heterologous antibodies of a switched isotype (i.e., IgG, IgA, and IgE).

          This invention provides methods whereby the transgene contains sequences that effectuate isotype

25   switching, so that the heterologous immunoglobulins produced in the transgenic animal and monoclonal antibody clones derived from the B-cells of said animal may be of various isotypes.

          This invention further provides methods that

30   facilitate isotype switching of the transgene, so that switching between particular isotypes may occur at much higher or lower frequencies or in different temporal orders than

typically occurs in germline immunoglobulin loci. Switch
regions may be grafted from various C_H genes and ligated to
other C_H genes in a transgene construct; such grafted switch
sequences will typically function independently of the

5 associated C_H gene so that switching in the transgene construct
will typically be a function of the origin of the associated
switch regions. Alternatively, or in combination with switch
sequences, δ-associated deletion sequences may be linked to
various C_H genes to effect non-classical switching by deletion

10 of sequences between two δ-associated deletion sequences.
Thus, a transgene may be constructed so that a particular C_H
gene is linked to a different switch sequence and thereby is
switched to more frequently than occurs when the naturally
associated switch region is used.

15 This invention also provides methods to determine
whether isotype switching of transgene sequences has occurred
in a transgenic animal containing an immunoglobulin transgene.

The invention provides immunoglobulin transgene
constructs and methods for producing immunoglobulin transgene

20 constructs, some of which contain a subset of germline
immunoglobulin loci sequences (which may include deletions).
The invention includes a specific method for facilitated
cloning and construction of immunoglobulin transgenes,
involving a vector that employs unique XhoI and SalI

25 restriction sites flanked by two unique NotI sites. This
method exploits the complementary termini of XhoI and SalI
restrictions sites and is useful for creating large constructs
by ordered concatemerization of restriction fragments in a
vector.

30 The transgenes of the invention include a heavy
chain transgene comprising DNA encoding at least one variable
gene segment, one diversity gene segment, one joining gene

segment and one constant region gene segment. The
immunoglobulin light chain transgene comprises DNA encoding at
least one variable gene segment, one joining gene segment and
one constant region gene segment. The gene segments encoding

5   the light and heavy chain gene segments are heterologous to
the transgenic non-human animal in that they are derived from,
or correspond to, DNA encoding immunoglobulin heavy and light
chain gene segments from a species not consisting of the
transgenic non-human animal. In one aspect of the invention,

10   the transgene is constructed such that the individual gene
segments are unrearranged, i.e., not rearranged so as to
encode a functional immunoglobulin light or heavy chain. Such
unrearranged transgenes permit recombination of the gene
segments (functional rearrangement) and expression of the

15   resultant rearranged immunoglobulin heavy and/or light chains
within the transgenic non-human animal when said animal is
exposed to antigen.

            In one aspect of the invention, heterologous heavy
and light immunoglobulin transgenes comprise relatively large

20   fragments of unrearranged heterologous DNA. Such fragments
typically comprise a substantial portion of the C, J (and in
the case of heavy chain, D) segments from a heterologous
immunoglobulin locus. In addition, such fragments also
comprise a substantial portion of the variable gene segments.

25   In one embodiment, such transgene constructs
comprise regulatory sequences, e.g. promoters, enhancers,
class switch regions, recombination signals and the like,
corresponding to sequences derived from the heterologous DNA.
Alternatively, such regulatory sequences may be incorporated

30   into the transgene from the same or a related species of the
non-human animal used in the invention. For example, human
immunoglobulin gene segments may be combined in a transgene

with a rodent immunoglobulin enhancer sequence for use in a
transgenic mouse.

In a method of the invention, a transgenic non-human
animal containing germline unrearranged light and heavy
5   immunoglobulin transgenes - that undergo VDJ joining during
D-cell differentiation - is contacted with an antigen to
induce production of a heterologous antibody in a secondary
repertoire B-cell.

Also included in the invention are vectors and
10   methods to disrupt the endogenous immunoglobulin loci in the
non-human animal to be used in the invention. Such vectors
and methods utilize a transgene, preferably positive-negative
selection vector, which is constructed such that it targets
the functional disruption of a class of gene segments encoding
15   a heavy and/or light immunoglobulin chain endogenous to the
non-human animal used in the invention. Such endogenous gene
segments include diversity, joining and constant region gene
segments. In this aspect of the invention, the
positive-negative selection vector is contacted with at least
20   one embryonic stem cell of a non-human animal after which
cells are selected wherein the positive-negative selection
vector has integrated into the genome of the non-human animal
by way of homologous recombination. After transplantation,
the resultant transgenic non-human animal is substantially
25   incapable of mounting an immunoglobulin-mediated immune
response as a result of homologous integration of the vector
into chromosomal DNA. Such immune deficient non-human animals
may thereafter be used for study of immune deficiencies or
used as the recipient of heterologous immunoglobulin heavy and
30   light chain transgenes.

The invention also provides vectors, methods, and
compositions useful for suppressing the expression of one or

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more species of immunoglobulin chain(s), without disrupting an
endogenous immunoglobulin locus. Such methods are useful for
suppressing expression of one or more endogenous
immunoglobulin chains while permitting the expression of one

5   or more transgene-encoded immunoglobulin chains. Unlike
genetic disruption of an endogenous immunoglobulin chain
locus, suppression of immunoglobulin chain expression does not
require the time-consuming breeding that is needed to
establish transgenic animals homozygous for a disrupted

10   endogenous Ig locus. An additional advantage of suppression
as compared to engognous Ig gene disruption is that, in
certain embodiments, chain suppression is reversible within an
individual animal. For example, Ig chain suppression may be
accomplished with: (1) transgenes encoding and expressing

15   antisense RNA that specifically hybridizes to an endogenous Ig
chain gene sequence, (2) antisense oligonucleotides that
specifically hybridize to an endogenous Ig chain gene
sequence, and (3) immunoglobulins that bind specifically to an
endogenous Ig chain polypeptide.

20   The invention provides transgenic non-human animals
comprising: a homozygous pair of functionally disrupted
endogenous heavy chain alleles, a homozygous pair of
functionally disrupted endogenous light chain alleles, at
least one copy of a heterologous immunoglobulin heavy chain

25   transgene, and at least one copy of a heterologous
immunoglobulin heavy chain transgene, wherein said animal
makes an antibody response following immunization with an
antigen, such as a human antigen (e.g., CD4). The invention
also provides such a transgenic non-human animal wherein said

30   functionally disrupted endogenous heavy chain allele is a J_H
region homologous recombination knockout, said functionally
disrupted endogenous light chain allele is a J_κ region

homologous recombination knockout, said heterologous
immunoglobulin heavy chain transgene is the HC1 or HC2 human
minigene transgene, said heterologous light chain transgene
is the KC2 or KC1e human κ transgene, and wherein said antigen
5   is a human antigen.

The invention also provides various embodiments for
suppressing, ablating, and/or functionally disrupting the
endogenous nonhuman immunoglobulin loci.

The invention also provides transgenic mice
10 expressing both human sequence heavy chains and chimeric heavy
chains comprising a human sequence heavy chain variable region
and a murine sequence heavy chain constant region. Such
chimeric heavy chains are generally produced by trans-
switching between a functionally rearranged human transgene
15 and an endogenous murine heavy chain constant region (e.g.,
γ1, γ2a, γ2b, γ3). Antibodies comprising such chimeric heavy
chains, typically in combination with a transgene-encoded
human sequence light chain or endogenous murine light chain,
are formed in response to immunization with a predetermined
20 antigen. The transgenic mice of these embodiments can
comprise B cells which produce (express) a human sequence
heavy chain at a first timepoint and trans-switch to produce
(express) a chimeric heavy chain composed of a human variable
region and a murine constant region (e.g., γ1, γ2a, γ2b, γ3)
25 at a second (subsequent) timepoint; such human sequence and
chimeric heavy chains are incorporated into functional
antibodies with light chains; such antibodies are present in
the serum of such transgenic mice. Thus, to restate: the
transgenic mice of these embodiments can comprise B cells
30 which express a human sequence heavy chain and subsequently
switch (via trans-switching or cis-switching) to express a
chimeric or isotype-switched heavy chain composed of a human

variable region and a alternative constant region (e.g.,
murine γ1, γ2a, γ2b, γ3; human γ, α, ε); such human sequence
and chimeric or isotype-switched heavy chains are incorporated
into functional antibodies with light chains (human or mouse);

5   such antibodies are present in the serum of such transgenic
mice.

The invention also provides a method for generating
a large transgene, said method comprising:

introducing into a mammalian cell at least three

10   polynucleotide species; a first polynucleotide species having
a recombinogenic region of sequence identity shared with a
second polynucleotide species, a second polynucleotide species
having a recombinogenic region of sequence identity shared
with a first polynucleotide species and a recombinogenic

15   region of sequence identity shared with a third polynucleotide
species, and a third polynucleotide species having a
recombinogenic region of sequence identity shared with said
second polynucleotide species.

Recombinogenic regions are regions of substantial

20   sequence identity sufficient to generate homologous
recombination in vivo in a mammalian cell (e.g., ES cell), and
preferably also in non-mammalian eukaryotic cells (e.g.,
Saccharaomyces and other yeast or fungal cells). Typically,
recombinogenic regions are at least 50 to 100000 nucleotides

25   long or longer, preferably 500 nucleotides to 10000
nucleotides long, and are often 80-100 percent identical,
frequently 95-100 percent identical, often isogenic.

The references discussed herein are provided solely
for their disclosure prior to the filing date of the present

30   application. Nothing herein is to be construed as an
admission that the inventors are not entitled to antedate such
disclosure by virtue of prior invention.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts the complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4 in unrearranged genomic DNA and mRNA expressed from a rearranged immunoglobulin heavy chain gene,

Fig. 2 depicts the human λ chain locus,

Fig. 3 depicts the human κ chain locus,

Fig. 4 depicts the human heavy chain locus,

Fig. 5 depicts a transgene construct containing a rearranged IgM gene ligated to a 25 kb fragment that contains human γ3 and γ1 constant regions followed by a 700 bp fragment containing the rat chain 3' enhancer sequence.

Fig. 6 is a restriction map of the human κ chain locus depicting the fragments to be used to form a light chain transgene by way of _in vivo_ homologous recombination.

Fig. 7 depicts the construction of pGP1.

Fig. 8 depicts the construction of the polylinker contained in pGP1.

Fig. 9 depicts the fragments used to construct a human heavy chain transgene of the invention.

Fig. 10 depicts the construction of pHIG1 and pCON1.

Fig. 11 depicts the human Cγ1 fragments which are inserted into pRE3 (rat enhancer 3') to form pREG2.

Fig. 12 depicts the construction of pHIG3' and PCON.

Fig. 13 depicts the fragment containing human D region segments used in construction of the transgenes of the invention.

Fig. 14 depicts the construction of pHIG2 (D segment containing plasmid).

Fig. 15 depicts the fragments covering the human Jκ and human Cκ gene segments used in constructing a transgene of the invention.

Fig. 16 depicts the structure of pEµ.

Fig. 17 depicts the construction of pKapH.

Figs. 18A through 18D depict the construction of a positive-negative selection vector for functionally disrupting the endogenous heavy chain immunoglobulin locus of mouse.

Figs. 19A through 19C depict the construction of a positive-negative selection vector for functionally disrupting the endogenous immunoglobulin light chain loci in mouse.

Figs. 20A through 20E depict the structure of a kappa light chain targeting vector.

Figs. 21A through 21F depict the structure of a mouse heavy chain targeting vector.

Fig. 22 depicts the map of vector pGPe.

Fig. 23 depicts the structure of vector pJM2.

Fig. 24 depicts the structure of vector pCOR1.

Fig. 25 depicts the transgene constructs for pIGM1, pHC1 and pHC2.

Fig. 26 depicts the structure of pγe2.

Fig. 27 depicts the structure of pVGE1.

Fig. 28 depicts the assay results of human Ig expression in a pHC1 transgenic mouse.

Fig. 29 depicts the structure of pJCK1.

Fig. 30 depicts the construction of a synthetic heavy chain variable region.

Fig. 31 is a schematic representation of the heavy chain minilocus constructs pIGM$_1$, pHC1, and pHC2.

Fig. 32 is a schematic representation of the heavy chain minilocus construct pIGG1 and the κ light chain minilocus construct pKC1, pKVe1, and pKC2.

Fig. 33 depicts a scheme to reconstruct functionally rearranged light chain genes.

Fig. 34 depicts serum ELISA results.

Fig. 35 depicts the results of an ELISA assay of serum from 8 transgenic mice.

Fig. 36 is a schematic representation of plasmid pBCE1.

5   Figs. 37A-37C depict the immune response of transgenic mice of the present invention against KLH-DNP, by measuring IgG and IgM levels specific for KLH-DNP (37A), KLH (37B) and BSA-DNP (37C).

Fig. 38 shows ELISA data demonstrating the presence

10   of antibodies that bind human carcinoembryonic antigen (CEA) and comprise human μ chains; each panel shows reciprocal serial dilutions from pooled serum samples obtained from mice on the indicated day following immunization.

Fig. 39 shows ELISA data demonstrating the presence

15   of antibodies that bind human carcinoembryonic antigen (CEA) and comprise human γ chains; each panel shows reciprocal serial dilutions from pooled serum samples obtained from mice on the indicated day following immunization.

Fig. 40 shows aligned variable region sequences of

20   23 randomly-chosen cDNAs generated from mRNA obtained from lymphoid tissue of HC1 transgenic mice immunized with human carcinoembryonic antigen (CEA) as compared to the germline transgene sequence (top line); on each line nucleotide changes relative to germline sequence are shown. The regions

25   corresponding to heavy chain CDR1, CDR2, and CDR3 are indicated. Non-germline encoded nucleotides are shown in capital letters.

Fig. 41 show the nucleotide sequence of a human DNA fragment, designated vk65.3, containing a V_x gene segment; the

30   deduced amino acid sequences of the V_x coding regions are also shown; splicing and recombination signal sequences (heptamer/nonamer) are shown boxed.

Fig. 42 show the nucleotide sequence of a human DNA fragment, designated vk65.5, containing a V_κ gene segment; the deduced amino acid sequences of the V_κ coding regions are also shown; splicing and recombination signal sequences

5   (heptamer/nonamer) are shown boxed.

Fig. 43 show the nucleotide sequence of a human DNA fragment, designated vk65.8, containing a V_κ gene segment; the deduced amino acid sequences of the V_κ coding regions are also shown; splicing and recombination signal sequences

10   (heptamer/nonamer) are shown boxed.

Fig. 44 show the nucleotide sequence of a human DNA fragment, designated vk65.15, containing a V_κ gene segment; the deduced amino acid sequences of the V_κ coding regions are also shown; splicing and recombination signal sequences

15   (heptamer/nonamer) are shown boxed.

Fig. 45 shows formation of a light chain minilocus by homologous recombination between two overlapping fragments which were co-injected.

Fig. 46 shows ELISA results for monoclonal

20   antibodies reactive with CEA and non-CEA antigens showing the specificity of antigen binding.

Fig. 47 shows the DNA sequences of 10 cDNAs amplified by PCR to amplify transcripts having a human VDJ and a murine constant region sequence.

25   Fig. 48 shows ELISA results for various dilutions of serum obtained from mice bearing both a human heavy chain minilocus transgene and a human κ minilocus transgene; the mouse was immunized with human CD4 and the data shown represents antibodies reactive with human CD4 and possessing

30   human κ, human μ, or human γ epitopes, respectively.

Fig. 49 shows relative distribution of lymphocytes staining for human μ or mouse μ as determined by FACS for three mouse genotypes.

Fig. 50 shows relative distribution of lymphocytes staining for human κ or mouse κ as determined by FACS for three mouse genotypes.

Fig. 51 shows relative distribution of lymphocytes staining for mouse λ as determined by FACS for three mouse genotypes.

Fig. 52 shows relative distribution of lymphocytes staining for mouse λ or human κ as determined by FACS for four mouse genotypes.

Fig. 53 shows the amounts of human μ, human γ, human κ, mouse μ, mouse γ, mouse κ, and mouse λ chains in the serum of unimmunized 0011 mice.

Fig. 54 shows a scatter plot showing the amounts of human μ, human γ, human κ, mouse μ, mouse γ, mouse κ, and mouse λ chains in the serum of unimmunized 0011 mice of various genotypes.

Fig. 55 shows the titres of antibodies comprising human μ, human γ, or human κ chains in anti-CD4 antibodies in the serum taken at three weeks or seven weeks post-immunization following immunization of a 0011 mouse with human CD4.

Fig. 56 shows a schematic representation of the human heavy chain minilocus transgenes pHC1 and pHC2, and the light chain minilocus transgenes pKC1, pKC1e, and the light chain minilocus transgene created by homologous recombination between pKC2 and Co4 at the site indicated.

Fig. 57 shows a linkage map of the murine lambda light chain locus as taken from Storb et al. (1989) op.cit.; the stippled boxes represent a pseudogene.

Fig. 58 shows a schematic representation of inactivation of the murine λ locus by homologous gene targeting.

Fig. 59 schematically shows the structure of a
5 homologous recombination targeting transgene for deleting genes, such as heavy chain constant region genes.

Fig. 60 shows a map of the BALB/c murine heavy chain locus as taken from Immunoglobulin Genes, Honjo, T, Alt, FW, and Rabbits TH (eds.) Academic Press, NY (1989) p. 129.
10 Structural genes are shown by closed boxes in the top line; second and third lines show restriction sites with symbols indicated.

Fig. 61 shows a nucleotide sequence of mouse heavy chain locus α constant region gene.
15   Fig. 62 shows the construction of a frameshift vector (plasmid B) for introducing a two bp frameshift into the murine heavy chain locus J_4 gene.

Fig. 63 shows isotype specific response of transgenic animals during hyperimmunization. The relative
20 levels of reactive human μ and γ1 are indicated by a colorimetric ELISA assay (y-axis). We immunized three 7-10 week old male HC1 line 57 transgenic animals (#1991, #2356, #2357), in a homozygous JHD background, by intraperitoneal injections of CEA in Freund's adjuvant. The figure depicts
25 binding of 250 fold dilutions of pooled serum (collected prior to each injection) to CEA coated microtiter wells.

Fig. 64A and 64B show expression of transgene encoded γ1 isotype mediated by class switch recombination. The genomic structure of integrated transgenes in two
30 different human γ1 expressing hybridomas is consistent with recombination between the μ and γ1 switch regions. Fig. 64A shows a Southern blot of PacI/SfiI digested DNA isolated from

three transgene expressing hybridomas. From left to right:
clone 92-09A-5H1-5, human γ1⁺/μ⁻; clone 92-90A-4G2-2, human
γ1⁺/μ⁻; clone 92-09A-4F7-A5-2, human γ1⁻,μ⁺. All three
hybridomas are derived from a 7 month old male mouse

5   hemizygous for the HC1-57 integration, and homozygous for the
JHD disruption (mouse #1991). The blot is hybridized with a
probe derived from a 2.3 kb BglII/SfiI DNA fragment spanning
the 3' half of the human γ1 switch region. No switch product
is found in the μ expressing hybridoma, while the two γ1

10   expressing hybridomas, 92-09A-5H1-5 and 92-09A-4G2-2, contain
switch products resulting in PacI/SfiI fragments of 5.1 and
5.3 kb respectively, Fig. 64B is a diagram of two possible
deletional mechanisms by which a class switch from μ to γ1 can
occur. The human μ gene is flanked by 400 bp direct repeats

15   (σμ and Σμ) which can recombine to delete μ. Class switching
by this mechanism will always generate a 6.4 kb PacI/SfiI
fragment, while class switching by recombination between the μ
and the γ1 switch regions will generate a PacI/SfiI fragment
between 4 and 7 kb, with size variation between individual

20   switch events. The two γ1 expressing hybridomas examined in
Fig. 64A appear to have undergone recombination between the μ
and γ1 switch regions.

          Fig. 65 shows chimeric human/mouse immunoglobulin
heavy chains generated by trans-switching. cDNA clones of

25   trans-switch products were generated by reverse transcription
and PCR amplification of a mixture of spleen and lymph node
RNA isolated from a hyperimmunized HC1 transgenic-JHD mouse
(#2357; see legend to Fig. 63 for description of animal and
immunization schedule). The partial nucleotide sequence of 10

30   randomly picked clones is shown. Lower case letters indicate
germline encoded, capital letters indicate nucleotides that
cannot be assigned to known germline sequences; these may be

somatic mutations, N nucleotides, or truncated D segments. Both face type indicates mouse γ sequences.

Figs. 66A and 66B show that the rearranged VH251 transgene undergoes somatic mutation in a hyperimmunized. The partial nucleotide sequence of IgG heavy chain variable region cDNA clones from CH1 line 26 mice exhibiting Fig. 66A primary and Fig. 66B secondary responses to antigen. Germline sequence is shown at the top; nucleotide changes from germline are given for each clone. A period indicates identity with germline sequence, capital letters indicate no identified germline origin. The sequences are grouped according to J segment usage. The germline sequence of each of the J segments if shown. Lower case letters within CDR3 sequences indicate identity to known D segment included in the HC1 transgene. The assigned D segments are indicated at the end of each sequence. Unassigned sequences could be derived from N region addition or somatic mutation; or in some cases they are simply too short to distinguish random N nucleotides from known D segments. Fig. 66A primary response: 13 randomly picked VH251-γ1 cDNA clones. A 4 week old female HC1 line 26-JHD mouse (#2599) was given a single injection of KLH and complete Freunds adjuvant; spleen cell RNA was isolated 5 days later. The overall frequency of somatic mutations within the V segment is 0.06% (2/3,198 bp). Fig. 66B secondary response: 13 randomly picked VH251-γ1 cDNA clones. A 2 month old female HC1 line 26-JHD mouse (#3204) was given 3 injections of HEL and Freunds adjuvant over one month (a primary injection with complete adjuvant and boosts with incomplete at one week and 3 weeks); spleen and lymph node RNA was isolated 4 months later. The overall frequency of somatic mutations within the V segment is 1.6% (52/3,198 bp).

Figs. 67A and 67B show that extensive somatic mutation is confined to γ1 sequences: somatic mutation and class switching occur within the same population of B cells. Partial nucleotide sequence of VH251 cDNA clones isolated from

5   spleen and lymph node cells of HC1 line 57 transgenic-JHD mouse (#2357) hyperimmunized against CEA (see Fig. 63 for immunization schedule). Fig. 67A: IgM: 23 randomly picked VH251-μ cDNA clones. Nucleotide sequence of 156 bp segment including CDRs 1 and 2 surrounding residues. The overall

10   level of somatic mutation is 0.1% (5/3,744 bp). Fig 67B: IgG: 23 randomly picked VH251-γ1 cDNA clones. Nucleotide sequence of segment including CDRs 1 through 3 and surrounding residues. The overall frequency of somatic mutation within the V segment is 1.1% (65/5,658 bp). For comparison with the

15   μ sequences in Fig. 67A: the mutation frequency for first 156 nucleotides is 1.1% (41/3,588 bp). See legend to Figs. 66A and 66B for explanation of symbols.

Fig. 68 indicates that VH51P1 and VH56P1 show extensive somatic mutation of in an unimmunized mouse. The

20   partial nucleotide sequence of IgG heavy chain variable region cDNA clones from a 9 week old, unimmunized female HC2 line 2550 transgenic-JHD mouse (#5250). The overall frequency of somatic mutation with the 19 VH56p1 segments is 2.2% (101/4,674 bp). The overall frequency of somatic mutation

25   within the single VH51p1 segment is 2.0% (5/246 bp). See legend to Figs. 66A and 66B for explanation of symbols.

Fig. 69. Double transgenic mice with disrupted endogenous Ig loci contain human IgMκ positive B cells. FACS of cells isolated from spleens of 4 mice with different

30   genotypes. Left column: control mouse (#9944, 6 wk old female JH+/-, JCκ+/-; heterozygous wild-type mouse heavy and κ-light chain loci, non-transgenic). Second column: human

heavy chain transgenic (#9877, 6 wk old female JH-/-, JCκ-/-,
HC2 line 2550 +; homozygous for disrupted mouse heavy and κ-
light chain loci, hemizygous for HC2 transgene). Third
column: human κ-light chain transgenic (#9878, 6 wk old

5   female JH-/-, JCκ-/-, KCo4 line 4437 +; homozygous for
disrupted mouse heavy and κ-light chain loci, hemizygous for
KCo4 transgene). Right column: double transgenic (#9879, 6
wk old female JH-/-m JCκ-/-, HC2 line 2550 +, KCo4 line 4437
+; homozygous for disrupted mouse heavy and κκ-light chain

10   loci, hemizygous for HC2 and KCo4 transgenes). Top row:
spleen cells stained for expression of mouse λ light chain (x-
axis) and human κ light chain (y-axis). Second row: spleen
cells stained for expression of human μ heavy chain (x-axis)
and human κ light chain (y-axis). Third row: spleen cells

15   stained for expression of mouse μ heavy chain (x-axis) and
mouse κ light chain (y-axis). Bottom row: histogram of
spleen cells stained for expression of mouse B220 antigen (log
fluorescence: x-axis; cell number: y-axis). For each of the
two color panels, the relative number of cells in each of the

20   displayed quadrants is given as percent of a e-parameter gate
based on propidium iodide staining and light scatter. The
fraction of B220+ cells in each of the samples displayed in
the bottom row is given as a percent of the lymphocyte light
scatter gate.

25   Fig. 70. Secreted immunoglobulin levels in the
serum of double transgenic mice. Human μ, γ, and κ, and mouse
γ and λ from 18 individual HC2/KCo4 double transgenic mice
homozygous for endogenous heavy and κ-light chain locus
disruption. Mice: (+) HC2 line 2550 (~5 copies of HC2 per

30   integration), KCo4 line 4436 (1-2 copies of KCo4 per
integration); (O) HC2 line 2550, KCo4 line 4437 (~10 copies of
KCo4 per integration); (x) HC2 line 2550, KCo4 line 4583 (~5

copies of KCo4 per integration); (□) HC2 line 2572 (30-50

copies of HC2 per integration, KCo4 line 4437; (Δ) HC2 line

5467 (20-30 copies of HC2 per integration, KCo4 line 4437.

Figs. 71A and 71B show human antibody responses to

5 human antigens. Fig. 71A: Primary response to recombinant

human soluble CD4. Levels of human IgM and human κ light

chain are reported for prebleed (O) and post-immunization (•)

serum from four double transgenic mice. Fig. 71B: Switching to

human IgG occurs in vivo. Human IgG (circles) was detected

10 with peroxidase conjugated polyclonal anti-human IgG used in

the presence of 1.5 μ/ml excess IgE, κ and 1% normal mouse

serum to inhibit non-specific cross-reactivity. Human κ light

chain (squares) was detected using a peroxidase conjugated

polyclonal anti-human κ reagent in the presence of 1% normal

15 mouse serum.   A representative result from one mouse (#9344;

HC2 line 2550, KCo4 line 4436) is shown. Each point

represents an average of duplicate wells minus background

absorbance.

Fig. 72 shows FACS analysis of human PBL with a

20 hybridoma supernatant that discriminates human CD4+

lymphocytes from human CD8+ lymphocytes.

Fig. 73 shows human α-CD4 IgM anf IgG in transgenic

mouse serum.

Fig. 74 shows competition binding experiments

25 comparing a transgenic mouse α-human CD4 hybridoma monoclonal,

2C11-8, to the RPA-TA and Leu-3A monoclonals.

Fig. 75 shows production data for Ig expression of

cultured 2C11-8 hybridoma.

Fig. 76 shows an overlapping set of plasmid inserts

30 constituting the HCo7 transgene.

Fig. 77A depicts the nucleotide sequence and

restriction map of pGP2b plasmid vector.

Fig. 77B depicts the restriction map of pGP2b plasmid vector.

Fig. 78 (parts A and B) depicts cloning strategy for assembling large transgenes.

Fig. 79 shows that large inserts are unstable in high-copy pUC derived plasmids.

Fig. 80 shows phage P1 clone P1-570. Insert spans portion of human heavy chain constant region covering γ3 and γ1, together with switch elements. N, NotI; S, SalI, X, XhoI.

Fig. 81 shows serum expression of human μ and γ1 in HCo7 transgenic founder animals.

Fig. 82 shows serum expression of human immunoglobulins in HCo7/KCo4 double transgenic/double deletion mice.

Fig. 83 shows RT PCR detection of human γ1 and γ3 transcripts in HCo7 transgenic mouse spleen RNA.

Fig. 84 shows induction of human IgG1 and IgG3 by LPS and IL-4 _in vitro_.

Fig. 85. Agarose gel electrophoresis apparatus for concentration of YAC DNA.

Fig. 86. Two color FACS analysis of bone marrow cells from HC2/KCo5/JHD/JKD and HC2/KCo4/JHD/JKD mice. The fraction of cells in each of the B220+/CD43-, B220+/CD43+, and B220+/IgM+ gates is given as a percent.

Fig. 87. Two color FACS analysis of spleen cells from HC2/KCo5/JHD/JKD and HC2/KCo4/JHD/JKD mice. The fraction of cells in each of the B220^{bright}/IgM+ and B220^{dull}/IgM+ gates is given as a percent.

Fig. 88. Binding of IgGκ anti-nCD4 monoclonal antibodies to CD4+ SupT1 cells.

Fig. 89 Epitope determination for IgG anti-nCD4 monoclonal antibodies by flow cytometry. SupT1 cells were pre-incubated with buffer

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(left column), 2.5 mg/ml RPA-T4 (middle column), or 2.5 mg/ml Leu3a (right column) and then with one of the 10 human IgG monoclonal antibodies (in supernatant diluted 1:2), or chimeric Leu3a. Results for 3 representative human IgG monoclonal antibodies are shown in this figure.

5   Fig. 90   Inhibition of an MLR by a human IgGk anti-CD4 monoclonal antibody.

               Figure 91 shows the effect of huMAb administration on CD4⁺ cells.

               Fig. 92 shows the effect of huMAb administration on CD4⁻ Cells.

10   Fig. 93 shows the effect of huMAb administration on cynomolgus monkey cells.

               Figure 94 shows the effect of huMAb administration on lymph node lymphocytes.

               Fig. 95 shows serum half-life of huMAbs in cynomolgus monkeys.

15   The data derived from 1E11 and 6G5 were fit to a two compartment model, whereas the data derived from 1G2 were fit to a one compartment model.

               Fig. 96 shows inhibition by human anti-CD4 mAbs of human cell responses to tetanus toxoid (TT). Panels (a) and (b) present results for two different assays. Lot numbers of the mAbs are shown in parantheses.

20   Fig. 97 shows human IgM and IgG anti-IL8 serum titers in transgenic mice. Responses of the individual mice to the immunogen were assessed by ELISA, converted to titers (1 = OD >0.1 at 1:50 dilution of serum; 2 = OD >0.1 at 1:250 dilution of serum; 3 = OD >0.1 at 1:1250 dilution of serum; 4 = OD >0.1 at 1:6250 dilution of serum; and 5 = OD >0.1

25   at 1:31250 dilution of serum) and then averaged.

               Fig. 98 shows the effect of human anti-IL8 mAb on IL8-induced neutrophil chemotaxis and elastase release.

               Table 1 depicts the sequence of vector pGPe.

               Table 2 depicts the sequence of gene V_H49.8.

30   Table 3 depicts the detection of human IgM and IgG in the serum of transgenic mice of this invention.

               Table 4 depicts sequences of VDJ joints.

               Table 5 depicts the distribution of J segments incorporated into pHC1 transgene encoded transcripts to J segments found in adult human

35   peripheral blood lymphocytes (PBL).

               Table 6 depicts the distribution of D segments incorporated into pHC1 transgene encoded transcripts to D segments found in adult human peripheral blood lymphocytes (PBL).

Table 7 depicts the length of the CDR3 peptides from transcripts with in-frame VDJ joints in the pHC1 transgenic mouse and in human PBL.

Table 8 depicts the predicted amino acid sequences of the VDJ regions from 30 clones analyzed from a pHC1 transgenic.

Table 9 shows transgenic mice of line 112 that were used in the indicated experiments; (+) indicates the presence of the respective transgene, (++) indicates that the animal is homozygous for the $J_H D$ knockout transgene.

Table 10 shows the genotypes of several 0011 mice.

Table 11 shows human variable region usage in hybridomas from transgenic mice.

Table 12 shows transgene V and J segment usage.

Table 13 shows the occurrence of somatic mutation in the HC2 heavy chain transgene in transgenic mice.

Table 14 shows identification of human V_K segments on the YAC 4x17E1.

Table 15. Identification of human vk genes expressed in mouse line KCo5-9272.

Table 16. Secretion levels for human IgGk Anti-nCD4 monoclonal antibodies

Table 17. Rate and affinity constants for monoclonal antibodies that bind to human CD4.

Table 18. Affinity and rate constants of human anti-human CD4 monoclonal antibodies.

Table 19. Avidity and rate constants of human anti-human CD4 monoclonal antibodies.

Table 20. Avidity and rate constants reported for anti CD4 monoclonal antibodies.

Table 21. Avidity constants of human anti-human CD4 monoclonal antibodies as determined by flow cytometry.

Table 22. Partial Nucleotide Sequence for Functional Transcripts.

Table 23 Germline V(D)J Segment Usage in Hybridoma Transcripts.

Table 24.   Primers, Vectors and Products Used in Minigene Construction.

Table 25.   Effect of Human mAbs on Peripheral Chimpanzee Lymphocytes.

Table 26.   Summary of Flow Cytometry Studies on Lymph Node Lymphocytes.

Table 27.   Monoclonal Antibody Secretion, Avidity and Rate Constants.

Table 28.   Specificity and Characterization of Human Anti-IL8 mAbs.

DETAILED DESCRIPTION

As has been discussed supra, it is desirable to produce human immunoglobulins that are reactive with specific human antigens that are promising therapeutic and/or diagnostic targets. However, producing human immunoglobulins that bind specifically with human antigens is problematic.

First, the immunized animal that serves as the source of B cells must make an immune response against the presented antigen. In order for an animal to make an immune response, the antigen presented must be foreign and the animal must not be tolerant to the antigen. Thus, for example, if it is desired to produce a human monoclonal antibody with an idiotype that binds to a human protein, self-tolerance will prevent an immunized human from making a substantial immune response to the human protein, since the only epitopes of the antigen that may be immunogenic will be those that result from polymorphism of the protein within the human population (allogeneic epitopes).

Second, if the animal that serves as the source of B-cells for forming a hybridoma (a human in the illustrative given example) does make an immune response against an

authentic self antigen, a severe autoimmune disease may result in the animal. Where humans would be used as a source of B-cells for a hybridoma, such autoimmunization would be considered unethical by contemporary standards. Thus,

5   developing hybridomas secreting human immunoglobulin chainsspecifically reactive with predetermined human antigens is problematic, since a reliable source of human antibody-secreting B cells that can evoke an antibody response against predetermined human antigens is needed.

10   One methodology that can be used to obtain human antibodies that are specifically reactive with human antigens is the production of a transgenic mouse harboring the human immunoglobulin transgene constructs of this invention. Briefly, transgenes containing all or portions of the human

15   immunoglobulin heavy and light chain loci, or transgenes containing synthetic "miniloci" (described *infra*, and in copending applications U.S.S.N. 08/352,322, filed 7 December 1994, U.S.S.N. 07/990,860, filed 16 December 1992, U.S.S.N. 07/810,279 filed 17 December 1991, U.S.S.N. 07/904,068 filed

20   23 June 1992; U.S.S.N. 07/853,408, filed 18 March 1992, U.S.S.N. 07/574,748 filed August 29, 1990, U.S.S.N. 07/575,962 filed August 31, 1990, and PCT/US91/06185 filed August 28, 1991, each incorporated herein by reference) which comprise essential functional elements of the human heavy and light

25   chain loci, are employed to produce a transgenic nonhuman animal. Such a transgenic nonhuman animal will have the capacity to produce immunoglobulin chains that are encoded by human immunoglobulin genes, and additionally will be capable of making an immune response against human antigens. Thus,

30   such transgenic animals can serve as a source of immune sera reactive with specified human antigens, and B-cells from such transgenic animals can be fused with myeloma cells to produce

hybridomas that secrete monoclonal antibodies that are encoded
by human immunoglobulin genes and which are specifically
reactive with human antigens.

The production of transgenic mice containing various
5   forms of immunoglobulin genes has been reported previously.
Rearranged mouse immunoglobulin heavy or light chain genes
have been used to produce transgenic mice. In addition,
functionally rearranged human Ig genes including the μ or $\gamma 1$
constant region have been expressed in transgenic mice.
10   However, experiments in which the transgene comprises
unrearranged (V-D-J or V-J not rearranged) immunoglobulin
genes have been variable, in some cases, producing incomplete
or minimal rearrangement of the transgene. However, there are
no published examples of either rearranged or unrearranged
15   immunoglobulin transgenes which undergo successful isotype
switching between C_H genes within a transgene.

The invention also provides a method for identifying
candidate hybridomas which secrete a monoclonal antibody
comprising a human immunoglobulin chain consisting essentially
20   of a human VDJ sequence in polypeptide linkage to a human
constant region sequence. Such candidate hybridomas are
identified from a pool of hybridoma clones comprising: (1)
hybridoma clones that express immunoglobulin chains consisting
essentially of a human VDJ region and a human constant region,
25   and (2) trans-switched hybridomas that express heterohybrid
immunoglobulin chains consisting essentially of a human VDJ
region and a murine constant region. The supernatant(s) of
individual or pooled hybridoma clones is contacted with a
predetermined antigen, typically an antigen which is
30   immobilized by adsoption onto a solid substrate (e.g., a
microtitre well), under binding conditions to select
antibodies having the predetermined antigen binding

specificity. An antibody that specifically binds to human
constant regions is also contacted with the hybridoma
supernatant and predetermined antigen under binding conditions
so that the antibody selectively binds to at least one human

5   constant region epitope but substantially does not bind to
murine constant region epitopes; thus forming complexes
consisting essentially of hybridoma supernatant (transgenic
monoclonal antibody) bound to a predetermined antigen and to
an antibody that specifically binds human constant regions

10   (and which may be labeled with a detectable label or
reporter). Detection of the formation of such complexes
indicates hybridoma clones or pools which express a human
immunoglobulin chain.

     In a preferred embodiment of the invention, the

15   anti-human constant region immunoglobulin used in screening
specifically recognizes a non-μ, non-δ isotype, preferably a α
or ϵ, more perferrably a γ isotype constant region.
Monoclonal antibodies of the γ isotype are preferred (i)
because the characteristics of IgG immunoglobulins are

20   preferable to IgM immunogloblins for some therapeutic
applications (e.g., due to the smaller size of the IgG dimers
compared to IgM pentamers) and, (ii) because the process of
somatic mutation is correlated with the class switch from the
μ constant region to the non-μ (e.g., γ) constant regions.

25   Immunoglobulins selected from the population of
immunoglobulins that have undergone class switch (e.g., IgG)
tend to bind antigen with higher affinity than immunoglobulins
selected from the population that has not undergone class
switch (e.g., IgM). See, e.g., Lonberg and Huszar. Intern.

30   Rev. Immunol. 13:65-93 (1995) which is incorporated herein by
reference.

In one embodiment the candidate hybridomas are first screened for the γ isotype constant region and the pool of IgG-expressing hybridomas is then screened for specific binding to the predetermined antigen.

5 Thus, according to the method, a transgenic mouse of the invention is immunized with the predetermined antigen to induce an immune response. B cells are collected from the mouse and fused to immortal cells to produce hybridomas. The hybridomas are first screened to identify individual 10 hybridomas secreting Ig of a non-mu, non-delta isotype (e..g., IgG). This set of hybridomas is then screened for specific binding to the predetermined antigen of interest. Screening is carried out using standard techniques as described in, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold 15 Spring Harbor, New York (1988). Using this method it is possible to identify high-affinity immunoglobulins (e.g., Ka greater than about 10^7 M^{-1}) practically and efficiently.

Definitions

20 As used herein, the term "antibody" refers to a glycoprotein comprising at least two light polypeptide chains and two heavy polypeptide chains. Each of the heavy and light polypeptide chains contains a variable region (generally the amino terminal portion of the polypeptide chain) which 25 contains a binding domain which interacts with antigen. Each of the heavy and light polypeptide chains also comprises a constant region of the polypeptide chains (generally the carboxyl terminal portion) which may mediate the binding of the immunoglobulin to host tissues or factors including 30 various cells of the immune system, some phagocytic cells and the first component (Clq) of the classical complement system.

As used herein, a "heterologous antibody" is defined in relation to the transgenic non-human organism producing such an antibody. It is defined as an antibody having an amino acid sequence or an encoding DNA sequence corresponding

5   to that found in an organism not consisting of the transgenic non-human animal, and generally from a species other than that of the transgenic non-human animal.

As used herein, a "heterohybrid antibody" refers to an antibody having a light and heavy chains of different

10 organismal origins. For example, an antibody having a human heavy chain associated with a murine light chain is a heterohybrid antibody.

As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG_1) that is encoded by heavy chain

15 constant region genes.

As used herein, "isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

As used herein, "nonswitched isotype" refers to the

20 isotypic class of heavy chain that is produced when no isotype switching has taken place; the C_H gene encoding the nonswitched isotype is typically the first C_H gene immediately downstream from the functionally rearranged VDJ gene.

As used herein, the term "switch sequence" refers to

25 those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, will be 5' (i.e., upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region will be between the construct region to be deleted and the

30 replacement constant region (e.g., γ, ϵ, etc.). As there is no specific site where recombination always occurs, the final

gene sequence will typically not be predictable from the construct.

As used herein, "glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin protein. A glycosylation pattern of a heterologous antibody can be characterized as being substantially similar to glycosylation patterns which occur naturally on antibodies produced by the species of the nonhuman transgenic animal, when one of ordinary skill in the art would recognize the glycosylation pattern of the heterologous antibody as being more similar to said pattern of glycosylation in the species of the nonhuman transgenic animal than to the species from which the C_H genes of the transgene were derived.

As used herein, "specific binding" refers to the property of the antibody: (1) to bind to a predetermined antigen with an affinity of at least 1×10^7 M^{-1}, and (2) to preferentially bind to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "rearranged" as used herein refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent

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to a D-J or J segment in a conformation encoding essentially a complete V_H or V_L domain, respectively. A rearranged immunoglobulin gene locus can be identified by comparison to germline DNA; a rearranged locus will have at least one

5   recombined heptamer/nonamer homology element.

The term "unrearranged" or "germline configuration" as used herein in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

10   For nucleic acids, the term "substantial homology" indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90%

15   to 95%, and more preferably at least about 98 to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a

20   partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding,

25   column chromatography, agarose gel electrophoresis and others well known in the art. See, F. Ausubel, et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1987).

The nucleic acid compositions of the present

30   invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures may be mutated, thereof in accordance with

standard techniques to provide gene sequences. For coding
sequences, these mutations, may affect amino acid sequence as
desired. In particular, DNA sequences substantially
homologous to or derived from native V, D, J, constant,
5   switches and other such sequences described herein are
contemplated (where "derived" indicates that a sequence is
identical or modified from another sequence).

        A nucleic acid is "operably linked" when it is
placed into a functional relationship with another nucleic
10   acid sequence. For instance, a promoter or enhancer is
operably linked to a coding sequence if it affects the
transcription of the sequence. With respect to transcription
regulatory sequences, operably linked means that the DNA
sequences being linked are contiguous and, where necessary to
15   join two protein coding regions, contiguous and in reading
frame. For switch sequences, operably linked indicates that
the sequences are capable of effecting switch recombination.

Transgenic Nonhuman Animals Capable
20   of Producing Heterologous Antibodies
        The design of a transgenic non-human animal that
responds to foreign antigen stimulation with a heterologous
antibody repertoire, requires that the heterologous
immunoglobulin transgenes contained within the transgenic
25   animal function correctly throughout the pathway of B-cell
development. In a preferred embodiment, correct function of a
heterologous heavy chain transgene includes isotype switching.
Accordingly, the transgenes of the invention are constructed
so as to produce isotype switching and one or more of the
30   following: (1) high level and cell-type specific expression,
(2) functional gene rearrangement, (3) activation of and
response to allelic exclusion, (4) expression of a sufficient

primary repertoire, (5) signal transduction, (6) somatic
hypermutation, and (7) domination of the transgene antibody
locus during the immune response.

    As will be apparent from the following disclosure,
5   not all of the foregoing criteria need be met. For example, in
those embodiments wherein the endogenous immunoglobulin loci
of the transgenic animal are functionally disrupted, the
transgene need not activate allelic exclusion. Further, in
those embodiments wherein the transgene comprises a
10 functionally rearranged heavy and/or light chain
immunoglobulin gene, the second criteria of functional gene
rearrangement is unnecessary, at least for that transgene
which is already rearranged. For background on molecular
immunology, see, Fundamental Immunology, 2nd edition (1989),
15 Paul William E., ed. Raven Press, N.Y., which is incorporated
herein by reference.

    In one aspect of the invention, transgenic non-human
animals are provided that contain rearranged, unrearranged or
a combination of rearranged and unrearranged heterologous
20 immunoglobulin heavy and light chain transgenes in the
germline of the transgenic animal. Each of the heavy chain
transgenes comprises at least one C_H gene. In addition, the
heavy chain transgene may contain functional isotype switch
sequences, which are capable of supporting isotype switching
25 of a heterologous transgene encoding multiple C_H genes in B-
cells of the transgenic animal. Such switch sequences may be
those which occur naturally in the germline immunoglobulin
locus from the species that serves as the source of the
transgene C_H genes, or such switch sequences may be derived
30 from those which occur in the species that is to receive the
transgene construct (the transgenic animal). For example, a
human transgene construct that is used to produce a transgenic

mouse may produce a higher frequency of isotype switching
events if it incorporates switch sequences similar to those
that occur naturally in the mouse heavy chain locus, as
presumably the mouse switch sequences are optimized to
5   function with the mouse switch recombinase enzyme system,
whereas the human switch sequences are not. Switch sequences
made be isolated and cloned by conventional cloning methods,
or may be synthesized de novo from overlapping synthetic
oligonucleotides designed on the basis of published sequence
10   information relating to immunoglobulin switch region sequences
(Mills et al., _Nucl. Acids Res_. _18_:7305-7316 (1991);
Sideras et al., _Intl. Immunol_. _1_:631-642 (1989), which are
incorporated herein by reference).

     For each of the foregoing transgenic animals,
15   functionally rearranged heterologous heavy and light chain
immunoglobulin transgenes are found in a significant fraction
of the B-cells of the transgenic animal (at least 10 percent).

     The transgenes of the invention include a heavy
chain transgene comprising DNA encoding at least one variable
20   gene segment, one diversity gene segment, one joining gene
segment and at least one constant region gene segment. The
immunoglobulin light chain transgene comprises DNA encoding at
least one variable gene segment, one joining gene segment and
at least one constant region gene segment. The gene segments
25   encoding the light and heavy chain gene segments are
heterologous to the transgenic non-human animal in that they
are derived from, or correspond to, DNA encoding
immunoglobulin heavy and light chain gene segments from a
species not consisting of the transgenic non-human animal. In
30   one aspect of the invention, the transgene is constructed such
that the individual gene segments are unrearranged, i.e., not
rearranged so as to encode a functional immunoglobulin light

or heavy chain. Such unrearranged transgenes support
recombination of the V, D, and J gene segments (functional
rearrangement) and preferably support incorporation of all or
a portion of a D region gene segment in the resultant
5   rearranged immunoglobulin heavy chain within the transgenic
non-human animal when exposed to antigen.

      In an alternate embodiment, the transgenes comprise
an unrearranged "mini-locus". Such transgenes typically
comprise a substantial portion of the C, D, and J segments as
10   well as a subset of the V gene segments. In such transgene
constructs, the various regulatory sequences, e.g. promoters,
enhancers, class switch regions, splice-donor and splice-
acceptor sequences for RNA processing, recombination signals
and the like, comprise corresponding sequences derived from
15   the heterologous DNA. Such regulatory sequences may be
incorporated into the transgene from the same or a related
species of the non-human animal used in the invention. For
example, human immunoglobulin gene segments may be combined in
a transgene with a rodent immunoglobulin enhancer sequence for
20   use in a transgenic mouse. Alternatively, synthetic regulatory
sequences may be incorporated into the transgene, wherein such
synthetic regulatory sequences are not homologous to a
functional DNA sequence that is known to occur naturally in
the genomes of mammals. Synthetic regulatory sequences are
25   designed according to consensus rules, such as, for example,
those specifying the permissible sequences of a splice-
acceptor site or a promoter/enhancer motif. For example, a
minilocus comprises a portion of the genomic immunoglobulin
locus having at least one internal (i.e., not at a terminus of
30   the portion) deletion of a non-essential DNA portion (e.g.,
intervening sequence; intron or portion thereof) as compared
to the naturally-occurring germline Ig locus.

The invention also includes transgenic animals
containing germ line cells having a heavy and light transgene
wherein one of the said transgenes contains rearranged gene
segments with the other containing unrearranged gene segments.
5   In the preferred embodiments, the rearranged transgene is a
light chain immunoglobulin transgene and the unrearranged
transgene is a heavy chain immunoglobulin transgene.

The Structure and Generation of Antibodies

10   The basic structure of all immunoglobulins is based
upon a unit consisting of two light polypeptide chains and two
heavy polypeptide chains. Each light chain comprises two
regions known as the variable light chain region and the
constant light chain region. Similarly, the immunoglobulin
15 heavy chain comprises two regions designated the variable
heavy chain region and the constant heavy chain region.

          The constant region for the heavy or light chain is
encoded by genomic sequences referred to as heavy or light
constant region gene (C_H) segments. The use of a particular
20 heavy chain gene segment defines the class of immunoglobulin.
For example, in humans, the μ constant region gene segments
define the IgM class of antibody whereas the use of a γ, $\gamma 2$,
$\gamma 3$ or $\gamma 4$ constant region gene segment defines the IgG class of
antibodies as well as the IgG subclasses IgG1 through IgG4.
25 Similarly, the use of a α_1 or α_2 constant region gene segment
defines the IgA class of antibodies as well as the subclasses
IgA1 and IgA2. The δ and ϵ constant region gene segments
define the IgD and IgE antibody classes, respectively.

          The variable regions of the heavy and light
30 immunoglobulin chains together contain the antigen binding
domain of the antibody. Because of the need for diversity in
this region of the antibody to permit binding to a wide range

of antigens, the DNA encoding the initial or primary
repertoire variable region comprises a number of different DNA
segments derived from families of specific variable region
gene segments. In the case of the light chain variable

5   region, such families comprise variable (V) gene segments and
joining (J) gene segments. Thus, the initial variable region
of the light chain is encoded by one V gene segment and one J
gene segment each selected from the family of V and J gene
segments contained in the genomic DNA of the organism. In the

10 case of the heavy chain variable region, the DNA encoding the
initial or primary repertoire variable region of the heavy
chain comprises one heavy chain V gene segment, one heavy
chain diversity (D) gene segment and one J gene segment, each
selected from the appropriate V, D and J families of

15 immunoglobulin gene segments in genomic DNA.

In order to increase the diversity of sequences that
contribute to forming antibody binding sites, it is preferable
that a heavy chain transgene include cis-acting sequences that
support functional V-D-J rearrangement that can incorporate

20 all or part of a D region gene sequence in a rearranged V-D-J
gene sequence. Typically, at least about 1 percent of
expressed transgene-encoded heavy chains (or mRNAs) include
recognizable D region sequences in the V region. Preferably,
at least about 10 percent of transgene-encoded V regions

25 include recognizable D region sequences, more preferably at
least about 30 percent, and most preferably more than 50
percent include recognizable D region sequences.

A recognizable D region sequence is generally at
least about eight consecutive nucleotides corresponding to a

30 sequence present in a D region gene segment of a heavy chain
transgene and/or the amino acid sequence encoded by such D
region nucleotide sequence. For example, if a transgene

includes the D region gene DHQ52, a transgene-encoded mRNA
containing the sequence 5'-TAACTGGG-3' located in the V region
between a V gene segment sequence and a J gene segment
sequence is recognizable as containing a D region sequence,

5   specifically a DHQ52 sequence. Similarly, for example, if a
transgene includes the D region gene DHQ52, a transgene-
encoded heavy chain polypeptide containing the amino acid
sequence -DAF- located in the V region between a V gene
segment amino acid sequence and a J gene segment amino acid

10   sequence may be recognizable as containing a D region
sequence, specifically a DHQ52 sequence. However, since D
region segments may be incorporated in VDJ joining to various
extents and in various reading frames, a comparison of the D
region area of a heavy chain variable region to the D region

15   segments present in the transgene is necessary to determine
the incorporation of particular D segments. Moreover,
potential exonuclease digestion during recombination may lead
to imprecise V-D and D-J joints during V-D-J recombination.

             However, because of somatic mutation and N-region

20   addition, some D region sequences may be recognizable but may
not correspond identically to a consecutive D region sequence
in the transgene. For example, a nucleotide sequence 5'-
CTAAXTGGGG-3', where X is A, T, or G, and which is located in
a heavy chain V region and flanked by a V region gene sequence

25   and a J region gene sequence, can be recognized as
corresponding to the DHQ52 sequence 5'-CTAACTGGG-3'.
Similarly, for example, the polypeptide sequences -DAFDI-,
-DYFDY-, or -GAFDI- located in a V region and flanked on the
amino-terminal side by an amino acid sequence encoded by a

30   transgene V gene sequence and flanked on the carboxyterminal
side by an amino acid sequence encoded by a transgene J gene
sequence is recognizable as a D region sequence.

Therefore, because somatic mutation and N-region addition can produce mutations in sequences derived from a transgene D region, the following definition is provided as a guide for determining the presence of a recognizable D region

5 sequence. An amino acid sequence or nucleotide sequence is recognizable as a D region sequence if: (1) the sequence is located in a V region and is flanked on one side by a V gene sequence (nucleotide sequence or deduced amino acid sequence) and on the other side by a J gene sequence (nucleotide

10 sequence or deduced amino acid sequence) and (2) the sequence is substantially identical or substantially similar to a known D gene sequence (nucleotide sequence or encoded amino acid sequence).

The term "substantial identity" as used herein

15 denotes a characteristic of a polypeptide sequence or nucleic acid sequence, wherein the polypeptide sequence has at least 50 percent sequence identity compared to a reference sequence, often at least about 80% sequence identity and sometimes more than about 90% sequence identity, and the nucleic acid

20 sequence has at least 70 percent sequence identity compared to a reference sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 35 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as an

25 entire D gene; however, the reference sequence is at least 8 nucleotides long in the case of polynucleotides, and at least 3 amino residues long in the case of a polypeptide. Typically, the reference sequence is at least 8 to 12 nucleotides or at least 3 to 4 amino acids, and preferably the

30 reference sequence is 12 to 15 nucleotides or more, or at least 5 amino acids.

The term "substantial similarity" denotes a characteristic of an polypeptide sequence, wherein the polypeptide sequence has at least 80 percent similarity to a reference sequence. The percentage of sequence similarity is

5   calculated by scoring identical amino acids or positional conservative amino acid substitutions as similar. A positional conservative amino acid substitution is one that can result from a single nucleotide substitution; a first amino acid is replaced by a second amino acid where a codon

10 for the first amino acid and a codon for the second amino acid can differ by a single nucleotide substitution. Thus, for example, the sequence -Lys-Glu-Arg-Val- is substantially similar to the sequence -Asn-Asp-Ser-Val-, since the codon sequence -AAA-GAA-AGA-GUU- can be mutated to -AAC-GAC-AGC-GUU-

15 by introducing only 3 substitution mutations, single nucleotide substitutions in three of the four original codons. The reference sequence may be a subset of a larger sequence, such as an entire D gene; however, the reference sequence is at least 4 amino residues long. Typically, the reference

20 sequence is at least 5 amino acids, and preferably the reference sequence is 6 amino acids or more.

The Primary Repertoire

        The process for generating DNA encoding the heavy

25 and light chain immunoglobulin genes occurs primarily in developing B-cells. Prior to the joining of various immunoglobulin gene segments, the V, D, J and constant (C) gene segments are found, for the most part, in clusters of V, D, J and C gene segments in the precursors of primary

30 repertoire B-cells. Generally, all of the gene segments for a heavy or light chain are located in relatively close proximity on a single chromosome. Such genomic DNA prior to

recombination of the various immunoglobulin gene segments is referred to herein as "unrearranged" genomic DNA. During B-cell differentiation, one of each of the appropriate family members of the V, D, J (or only V and J in the case of light

5   chain genes) gene segments are recombined to form functionally rearranged heavy and light immunoglobulin genes. Such functional rearrangement is of the variable region segments to form DNA encoding a functional variable region. This gene segment rearrangement process appears to be sequential.

10 First, heavy chain D-to-J joints are made, followed by heavy chain V-to-DJ joints and light chain V-to-J joints. The DNA encoding this initial form of a functional variable region in a light and/or heavy chain is referred to as "functionally rearranged DNA" or "rearranged DNA". In the case of the heavy

15 chain, such DNA is referred to as "rearranged heavy chain DNA" and in the case of the light chain, such DNA is referred to as "rearranged light chain DNA". Similar language is used to describe the functional rearrangement of the transgenes of the invention.

20   The recombination of variable region gene segments to form functional heavy and light chain variable regions is mediated by recombination signal sequences (RSS's) that flank recombinationally competent V, D and J segments. RSS's necessary and sufficient to direct recombination, comprise a

25 dyad-symmetric heptamer, an AT-rich nonamer and an intervening spacer region of either 12 or 23 base pairs. These signals are conserved among the different loci and species that carry out D-J (or V-J) recombination and are functionally interchangeable. See Oettinger, et al. (1990), Science, 248,

30 1517-1523 and references cited therein. The heptamer comprises the sequence CACAGTG or its analogue followed by a spacer of unconserved sequence and then a nonamer having the

sequence ACAAAAACC or its analogue. These sequences are found on the J, or downstream side, of each V and D gene segment. Immediately preceding the germline D and J segments are again two recombination signal sequences, first the nonamer and then

5 the heptamer again separated by an unconserved sequence. The heptameric and nonameric sequences following a V_L, V_H or D segment are complementary to those preceding the J_L, D or J_H segments with which they recombine. The spacers between the heptameric and nonameric sequences are either 12 base pairs

10 long or between 22 and 24 base pairs long.

In addition to the rearrangement of V, D and J segments, further diversity is generated in the primary repertoire of immunoglobulin heavy and light chain by way of variable recombination between the V and J segments in the

15 light chain and between the D and J segments of the heavy chain. Such variable recombination is generated by variation in the exact place at which such segments are joined. Such variation in the light chain typically occurs within the last codon of the V gene segment and the first codon of the J

20 segment. Similar imprecision in joining occurs on the heavy chain chromosome between the D and J_H segments and may extend over as many as 10 nucleotides. Furthermore, several nucleotides may be inserted between the D and J_H and between the V_H and D gene segments which are not encoded by genomic

25 DNA. The addition of these nucleotides is known as N-region diversity.

After VJ and/or VDJ rearrangement, transcription of the rearranged variable region and one or more constant region gene segments located downstream from the rearranged variable

30 region produces a primary RNA transcript which upon appropriate RNA splicing results in an mRNA which encodes a full length heavy or light immunoglobulin chain. Such heavy

and light chains include a leader signal sequence to effect
secretion through and/or insertion of the immunoglobulin into
the transmembrane region of the B-cell. The DNA encoding this
signal sequence is contained within the first exon of the V
5   segment used to form the variable region of the heavy or light
immunoglobulin chain. Appropriate regulatory sequences are
also present in the mRNA to control translation of the mRNA to
produce the encoded heavy and light immunoglobulin
polypeptides which upon proper association with each other
10 form an antibody molecule.

        The net effect of such rearrangements in the
variable region gene segments and the variable recombination
which may occur during such joining, is the production of a
primary antibody repertoire. Generally, each B-cell which has
15 differentiated to this stage, produces a single primary
repertoire antibody. During this differentiation process,
cellular events occur which suppress the functional
rearrangement of gene segments other than those contained
within the functionally rearranged Ig gene. The process by
20 which diploid B-cells maintain such mono-specificity is termed
allelic exclusion.

The Secondary Repertoire

        B-cell clones expressing immunoglobulins from within
25 the set of sequences comprising the primary repertoire are
immediately available to respond to foreign antigens. Because
of the limited diversity generated by simple VJ and VDJ
joining, the antibodies produced by the so-called primary
response are of relatively low affinity. Two different types
30 of B-cells make up this initial response: precursors of
primary antibody-forming cells and precursors of secondary
repertoire B-cells (Linton et al., Cell 59:1049-1059 (1989)).

The first type of B-cell matures into IgM-secreting plasma cells in response to certain antigens. The other B-cells respond to initial exposure to antigen by entering a T-cell dependent maturation pathway.

During the T-cell dependent maturation of antigen stimulated B-cell clones, the structure of the antibody molecule on the cell surface changes in two ways: the constant region switches to a non-IgM subtype and the sequence of the variable region can be modified by multiple single amino acid substitutions to produce a higher affinity antibody molecule.

As previously indicated, each variable region of a heavy or light Ig chain contains an antigen binding domain. It has been determined by amino acid and nucleic acid sequencing that somatic mutation during the secondary response occurs throughout the V region including the three complementary determining regions (CDR1, CDR2 and CDR3) also referred to as hypervariable regions 1, 2 and 3 (Kabat et al. Sequences of Proteins of Immunological Interest (1991) U.S. Department of Health and Human Services, Washington, DC, incorporated herein by reference. The CDR1 and CDR2 are located within the variable gene segment whereas the CDR3 is largely the result of recombination between V and J gene segments or V, D and J gene segments. Those portions of the variable region which do not consist of CDR1, 2 or 3 are commonly referred to as framework regions designated FR1, FR2, FR3 and FR4. See Fig. 1. During hypermutation, the rearranged DNA is mutated to give rise to new clones with altered Ig molecules. Those clones with higher affinities for the foreign antigen are selectively expanded by helper T-cells, giving rise to affinity maturation of the expressed antibody. Clonal selection typically results in expression of clones containing new mutation within the CDR1, 2 and/or 3

regions. However, mutations outside these regions also occur
which influence the specificity and affinity of the antigen
binding domain.

5   Transgenic Non-Human Animals Capable
    of Producing Heterologous Antibody

         Transgenic non-human animals in one aspect of the
    invention are produced by introducing at least one of the
10 immunoglobulin transgenes of the invention (discussed
    hereinafter) into a zygote or early embryo of a non-human
    animal. The non-human animals which are used in the invention
    generally comprise any mammal which is capable of rearranging
    immunoglobulin gene segments to produce a primary antibody
15 response. Such nonhuman transgenic animals may include, for
    example, transgenic pigs, transgenic rats, transgenic rabbits,
    transgenic cattle, and other transgenic animal species,
    particularly mammalian species, known in the art. A
    particularly preferred non-human animal is the mouse or other
20 members of the rodent family.

         However, the invention is not limited to the use of
    mice. Rather, any non-human mammal which is capable of
    mounting a primary and secondary antibody response may be
    used. Such animals include non-human primates, such as
25 chimpanzee, bovine, ovine, and porcine species, other members
    of the rodent family, e.g. rat, as well as rabbit and guinea
    pig. Particular preferred animals are mouse, rat, rabbit and
    guinea pig, most preferably mouse.

         In one embodiment of the invention, various gene
30 segments from the human genome are used in heavy and light
    chain transgenes in an unrearranged form. In this embodiment,
    such transgenes are introduced into mice. The unrearranged
    gene segments of the light and/or heavy chain transgene have

51

DNA sequences unique to the human species which are
distinguishable from the endogenous immunoglobulin gene
segments in the mouse genome. They may be readily detected in
unrearranged form in the germ line and somatic cells not
5 consisting of B-cells and in rearranged form in B-cells.

In an alternate embodiment of the invention, the
transgenes comprise rearranged heavy and/or light
immunoglobulin transgenes. Specific segments of such
transgenes corresponding to functionally rearranged VDJ or VJ
10 segments, contain immunoglobulin DNA sequences which are also
clearly distinguishable from the endogenous immunoglobulin
gene segments in the mouse.

Such differences in DNA sequence are also reflected
in the amino acid sequence encoded by such human
15 immunoglobulin transgenes as compared to those encoded by
mouse B-cells. Thus, human immunoglobulin amino acid
sequences may be detected in the transgenic non-human animals
of the invention with antibodies specific for immunoglobulin
epitopes encoded by human immunoglobulin gene segments.

20 Transgenic B-cells containing unrearranged
transgenes from human or other species functionally recombine
the appropriate gene segments to form functionally rearranged
light and heavy chain variable regions. It will be readily
apparent that the antibody encoded by such rearranged
25 transgenes has a DNA and/or amino acid sequence which is
heterologous to that normally encountered in the nonhuman
animal used to practice the invention.

Unrearranged Transgenes

30 As used herein, an "unrearranged immunoglobulin
heavy chain transgene" comprises DNA encoding at least one
variable gene segment, one diversity gene segment, one joining

gene segment and one constant region gene segment. Each of
the gene segments of said heavy chain transgene are derived
from, or has a sequence corresponding to, DNA encoding
immunoglobulin heavy chain gene segments from a species not

5 consisting of the non-human animal into which said transgene
is introduced. Similarly, as used herein, an "unrearranged
immunoglobulin light chain transgene" comprises DNA encoding
at least one variable gene segment, one joining gene segment
and at least one constant region gene segment wherein each

10 gene segment of said light chain transgene is derived from, or
has a sequence corresponding to, DNA encoding immunoglobulin
light chain gene segments from a species not consisting of the
non-human animal into which said light chain transgene is
introduced.

15   Such heavy and light chain transgenes in this aspect
of the invention contain the above-identified gene segments in
an unrearranged form. Thus, interposed between the V, D and J
segments in the heavy chain transgene and between the V and J
segments on the light chain transgene are appropriate

20 recombination signal sequences (RSS's). In addition, such
transgenes also include appropriate RNA splicing signals to
join a constant region gene segment with the VJ or VDJ
rearranged variable region.

          In order to facilitate isotype switching within a

25 heavy chain transgene containing more than one C region gene
segment, e.g. $C\mu$ and $C\gamma1$ from the human genome, as explained
below "switch regions" are incorporated upstream from each of
the constant region gene segments and downstream from the
variable region gene segments to permit recombination between

30 such constant regions to allow for immunoglobulin class
switching, e.g. from IgM to IgG. Such heavy and light
immunoglobulin transgenes also contain transcription control

sequences including promoter regions situated upstream from the variable region gene segments which typically contain TATA motifs. A promoter region can be defined approximately as a DNA sequence that, when operably linked to a downstream

5   sequence, can produce transcription of the downstream sequence. Promoters may require the presence of additional linked cis-acting sequences in order to produce efficient transcription. In addition, other sequences that participate in the transcription of sterile transcripts are preferably

10   included. Examples of sequences that participate in expression of sterile transcripts can be found in the published literature, including Rothman et al., _Intl. Immunol._ 2:621-627 (1990); Reid et al., _Proc. Natl. Acad. Sci. USA_ 86:840-844 (1989); Stavnezer et al., _Proc. Natl. Acad. Sci._

15   _USA_ 85:7704-7708 (1988); and Mills et al., _Nucl. Acids Res._ 18:7305-7316 (1991), each of which is incorporated herein by reference. These sequences typically include about at least 50 bp immediately upstream of a switch region, preferably about at least 200 bp upstream of a switch region; and more

20   preferably about at least 200-1000 bp or more upstream of a switch region. Suitable sequences occur immediately upstream of the human $S_{\gamma 1}$, $S_{\gamma 2}$, $S_{\gamma 3}$, $S_{\gamma 4}$, $S_{\alpha 1}$, $S_{\alpha 2}$, and S_{ϵ} switch regions; the sequences immediately upstream of the human $S_{\gamma 1}$, and $S_{\gamma 3}$ switch regions can be used to advantage, with $S_{\gamma 1}$ generally

25   preferred. Alternatively, or in combination, murine Ig switch sequences may be used; it may frequently be advantageous to employ Ig switch sequences of the same species as the transgenic non-human animal. Furthermore, interferon (IFN) inducible transcriptional regulatory elements, such as IFN-

30   inducible enhancers, are preferably included immediately upstream of transgene switch sequences.

     In addition to promoters, other regulatory sequences which function primarily in B-lineage cells are used. Thus, for example, a light chain enhancer sequence situated preferably between the J and constant region gene segments on

5   the light chain transgene is used to enhance transgene expression, thereby facilitating allelic exclusion. In the case of the heavy chain transgene, regulatory enhancers and also employed. Such regulatory sequences are used to maximize the transcription and translation of the transgene so as to

10   induce allelic exclusion and to provide relatively high levels of transgene expression.

     Although the foregoing promoter and enhancer regulatory control sequences have been generically described, such regulatory sequences may be heterologous to the nonhuman

15   animal being derived from the genomic DNA from which the heterologous transgene immunoglobulin gene segments are obtained. Alternately, such regulatory gene segments are derived from the corresponding regulatory sequences in the genome of the non-human animal, or closely related species,

20   which contains the heavy and light transgene.

     In the preferred embodiments, gene segments are derived from human beings. The transgenic non-human animals harboring such heavy and light transgenes are capable of mounting an Ig-mediated immune response to a specific antigen

25   administered to such an animal. B-cells are produced within such an animal which are capable of producing heterologous human antibody. After immortalization, and the selection for an appropriate monoclonal antibody (Mab), e.g. a hybridoma, a source of therapeutic human monoclonal antibody is provided.

30   Such human Mabs have significantly reduced immunogenicity when therapeutically administered to humans.

Although the preferred embodiments disclose the construction of heavy and light transgenes containing human gene segments, the invention is not so limited. In this regard, it is to be understood that the teachings described herein may be readily adapted to utilize immunoglobulin gene segments from a species other than human beings. For example, in addition to the therapeutic treatment of humans with the antibodies of the invention, therapeutic antibodies encoded by appropriate gene segments may be utilized to generate monoclonal antibodies for use in the veterinary sciences.

Rearranged Transgenes

In an alternative embodiment, transgenic nonhuman animals contain functionally at least one rearranged heterologous heavy chain immunoglobulin transgene in the germline of the transgenic animal. Such animals contain primary repertoire B-cells that express such rearranged heavy transgenes. Such B-cells preferably are capable of undergoing somatic mutation when contacted with an antigen to form a heterologous antibody having high affinity and specificity for the antigen. Said rearranged transgenes will contain at least two C_H genes and the associated sequences required for isotype switching.

The invention also includes transgenic animals containing germ line cells having heavy and light transgenes wherein one of the said transgenes contains rearranged gene segments with the other containing unrearranged gene segments. In such animals, the heavy chain transgenes shall have at least two C_H genes and the associated sequences required for isotype switching.

The invention further includes methods for generating a synthetic variable region gene segment repertoire

to be used in the transgenes of the invention. The method
comprises generating a population of immunoglobulin V segment
DNAs wherein each of the V segment DNAs encodes an
immunoglobulin V segment and contains at each end a cleavage
5   recognition site of a restriction endonuclease. The
population of immunoglobulin V segment DNAs is thereafter
concatenated to form the synthetic immunoglobulin V segment
repertoire. Such synthetic variable region heavy chain
transgenes shall have at least two C_H genes and the associated
10 sequences required for isotype switching.

Isotype Switching

In the development of a B lymphocyte, the cell
initially produces IgM with a binding specificity determined
15 by the productively rearranged V_H and V_L regions.
Subsequently, each B cell and its progeny cells synthesize
antibodies with the same L and H chain V regions, but they may
switch the isotype of the H chain.

The use of μ or δ constant regions is largely
20 determined by alternate splicing, permitting IgM and IgD to be
coexpressed in a single cell. The other heavy chain isotypes
(γ, α, and ϵ) are only expressed natively after a gene
rearrangement event deletes the $C\mu$ and $C\delta$ exons. This gene
rearrangement process, termed isotype switching, typically
25 occurs by recombination between so called switch segments
located immediately upstream of each heavy chain gene (except
δ). The individual switch segments are between 2 and 10 kb in
length, and consist primarily of short repeated sequences.
The exact point of recombination differs for individual class
30 switching events. Investigations which have used solution
hybridization kinetics or Southern blotting with cDNA-derived

C_H probes have confirmed that switching can be associated with loss of C_H sequences from the cell.

The switch (S) region of the μ gene, S_μ, is located about 1 to 2 kb 5' to the coding sequence and is composed of

5   numerous tandem repeats of sequences of the form $(GAGCT)_n(GGGGT)$, where n is usually 2 to 5 but can range as high as 17. (See T. Nikaido et al. *Nature* 292:845-848 (1981))

Similar internally repetitive switch sequences spanning several kilobases have been found 5' of the other C_H

10 genes. The S_α region has been sequenced and found to consist of tandemly repeated 80-bp homology units, whereas murine $S_{\gamma 2a}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ all contain repeated 49-bp homology units very similar to each other. (See, P. Szurek et al., *J. Immunol* 135:620-626 (1985) and T. Nikaido et al., *J. Biol. Chem.*

15 257:7322-7329 (1982), which are incorporated herein by reference.) All the sequenced S regions include numerous occurrences of the pentamers GAGCT and GGGGT that are the basic repeated elements of the S_μ gene (T. Nikaido et al., *J. Biol. Chem.* 257:7322-7329 (1982) which is incorporated herein

20 by reference); in the other S regions these pentamers are not precisely tandemly repeated as in S_μ, but instead are embedded in larger repeat units. The $S_{\gamma 1}$ region has an additional higher-order structure: two direct repeat sequences flank each of two clusters of 49-bp tandem repeats. (See M. R.

25 Mowatt et al., *J. Immunol.* 136:2674-2683 (1986), which is incorporated herein by reference).

Switch regions of human H chain genes have been found to be very similar to their mouse homologs. Indeed, similarity between pairs of human and mouse clones 5' to the C_H

30 genes has been found to be confined to the S regions, a fact that confirms the biological significance of these regions.

A switch recombination between μ and α genes produces a composite S_μ-S_α sequence. Typically, there is no specific site, either in S_μ or in any other S region, where the recombination always occurs.

5   Generally, unlike the enzymatic machinery of V-J recombination, the switch machinery can apparently accommodate different alignments of the repeated homologous regions of germline S precursors and then join the sequences at different positions within the alignment. (See, T. H. Rabbits et al.,

10 Nucleic Acids Res. 9:4509-4524 (1981) and J. Ravetch et al., Proc. Natl. Acad. Sci. USA 77:6734-6738 (1980), which are incorporated herein by reference.)

The exact details of the mechanism(s) of selective activation of switching to a particular isotype are unknown.

15 Although exogenous influences such as lymphokines and cytokines might upregulate isotype-specific recombinases, it is also possible that the same enzymatic machinery catalyzes switches to all isotypes and that specificity lies in targeting this machinery to specific switch regions.

20   The T-cell-derived lymphokines IL-4 and IFN$_\gamma$ have been shown to specifically promote the expression of certain isotypes: in the mouse, IL-4 decreases IgM, IgG2a, IgG2b, and IgG3 expression and increases IgE and IgG1 expression; while IFN$_\gamma$ selectively stimulates IgG2a expression and antagonizes

25 the IL-4-induced increase in IgE and IgG1 expression (Coffman et al., J. Immunol. 136: 949 (1986) and Snapper et al., Science 236: 944 (1987), which are incorporated herein by reference). A combination of IL-4 and IL-5 promotes IgA expression (Coffman et al., J. Immunol. 139: 3685 (1987),

30 which is incorporated herein by reference).

Most of the experiments implicating T-cell effects on switching have not ruled out the possibility that the

observed increase in cells with particular switch recombinations might reflect selection of preswitched or precommitted cells; but the most likely explanation is that the lymphokines actually promote switch recombination.

5   Induction of class switching appears to be associated with sterile transcripts that initiate upstream of the switch segments (Lutzker et al., _Mol. Cell. Biol._ _8_:1849 (1988); Stavnezer et al., _Proc. Natl. Acad. Sci. USA_ _85_:7704 (1988); Esser and Radbruch, _EMBO J._ _8_:483 (1989); Berton et

10   al., _Proc. Natl. Acad. Sci. USA_ _86_:2829 (1989); Rothman et al., _Int. Immunol._ _2_:621 (1990), each of which is incorporated by reference). For example, the observed induction of the γ1 sterile transcript by IL-4 and inhibition by IFN-γ correlates with the observation that IL-4 promotes class switching to γ1

15   in B-cells in culture, while IFN-γ inhibits γ1 expression. Therefore, the inclusion of regulatory sequences that affect the transcription of sterile transcripts may also affect the rate of isotype switching. For example, increasing the transcription of a particular sterile transcript typically can

20   be expected to enhance the frequency of isotype switch recombination involving adjacent switch sequences.

          For these reasons, it is preferable that transgenes incorporate transcriptional regulatory sequences within about 1-2 kb upstream of each switch region that is to be utilized

25   for isotype switching. These transcriptional regulatory sequences preferably include a promoter and an enhancer element, and more preferably include the 5' flanking (i.e., upstream) region that is naturally associated (i.e., occurs in germline configuration) with a switch region. This 5'

30   flanking region is typically about at least 50 nucleotides in length, preferably about at least 200 nucleotides in length, and more preferably at least 500-1000 nucleotides.

Although a 5' flanking sequence from one switch
region can be operably linked to a different switch region for
transgene construction (e.g., a 5' flanking sequence from the
human $S_{\gamma 1}$ switch can be grafted immediately upstream of the $S_{\alpha 1}$
5   switch; a murine $S_{\gamma 1}$ flanking region can be grafted adjacent to
a human $\gamma 1$ switch sequence; or the murine $S_{\gamma 1}$ switch can be
grafted onto the human $\gamma 1$ coding region), in some embodiments
it is preferred that each switch region incorporated in the
transgene construct have the 5' flanking region that occurs
10   immediately upstream in the naturally occurring germline
configuration.

Monoclonal Antibodies

Monoclonal antibodies can be obtained by various
15   techniques familiar to those skilled in the art. Briefly,
spleen cells from an animal immunized with a desired antigen
are immortalized, commonly by fusion with a myeloma cell (see,
Kohler and Milstein, Eur. J. Immunol., 6:511-519 (1976)).
Alternative methods of immortalization include transformation
20   with Epstein Barr Virus, oncogenes, or retroviruses, or other
methods well known in the art. Colonies arising from single
immortalized cells are screened for production of antibodies
of the desired specificity and affinity for the antigen, and
yield of the monoclonal antibodies produced by such cells may
25   be enhanced by various techniques, including injection into
the peritoneal cavity of a vertebrate host. Various
techniques useful in these arts are discussed, for example, in
Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring
Harbor, New York (1988) including: immunization of animals to
30   produce immunoglobulins; production of monoclonal antibodies;
labeling immunoglobulins for use as probes; immunoaffinity
purification; and immunoassays.

The Transgenic Primary Repertoire

A.   The Human Immunoglobulin Loci

      An important requirement for transgene function is
the generation of a primary antibody repertoire that is

5   diverse enough to trigger a secondary immune response for a
wide range of antigens. The rearranged heavy chain gene
consists of a signal peptide exon, a variable region exon and
a tandem array of multi-domain constant region regions, each
of which is encoded by several exons. Each of the constant

10 region genes encode the constant portion of a different class
of immunoglobulins. During B-cell development, V region
proximal constant regions are deleted leading to the
expression of new heavy chain classes. For each heavy chain
class, alternative patterns of RNA splicing give rise to both

15 transmembrane and secreted immunoglobulins.

      The human heavy chain locus is estimated to consist
of approximately 200 V gene segments (current data supports
the existence of about 50-100 V gene segments) spanning 2 Mb,
approximately 30 D gene segments spanning about 40 kb, six J

20 segments clustered within a 3 kb span, and nine constant
region gene segments spread out over approximately 300 kb.
The entire locus spans approximately 2.5 Mb of the distal
portion of the long arm of chromosome 14.

25 B.   Gene Fragment Transgenes

      1.   Heavy Chain Transgene

      In a preferred embodiment, immunoglobulin heavy and
light chain transgenes comprise unrearranged genomic DNA from
humans. In the case of the heavy chain, a preferred transgene

30 comprises a NotI fragment having a length between 670 to 830
kb. The length of this fragment is ambiguous because the 3'
restriction site has not been accurately mapped. It is known,

however, to reside between the α1 and ψα gene segments. This
fragment contains members of all six of the known V_H families,
the D and J gene segments, as well as the μ, δ, γ3, γ1 and α1
constant regions (Berman et al., EMBO J. 7:727-738 (1988),

5   which is incorporated herein by reference). A transgenic
mouse line containing this transgene correctly expresses a
heavy chain class required for B-cell development (IgM) and at
least one switched heavy chain class (IgG_1), in conjunction
with a sufficiently large repertoire of variable regions to

10   trigger a secondary response for most antigens.

        2.   Light Chain Transgene

            A genomic fragment containing all of the necessary
gene segments and regulatory sequences from a human light

15   chain locus may be similarly constructed. Such transgenes are
constructed as described in the Examples and in copending
application, entitled "Transgenic Non-Human Animals Capable
of Producing Heterologous Antibodies," filed August 29, 1990,
under U.S.S.N. 07/574,748.

20

C.   Transgenes Generated Intracellularly
     by In Vivo Recombination

            It is not necessary to isolate the all or part of
the heavy chain locus on a single DNA fragment. Thus, for

25   example, the 670-830 kb NotI fragment from the human
immunoglobulin heavy chain locus may be formed in vivo in the
non-human animal during transgenesis. Such in vivo transgene
construction is produced by introducing two or more
overlapping DNA fragments into an embryonic nucleus of the

30   non-human animal. The overlapping portions of the DNA
fragments have DNA sequences which are substantially
homologous. Upon exposure to the recombinases contained

within the embryonic nucleus, the overlapping DNA fragments homologously recombined in proper orientation to form the 670-830 kb NotI heavy chain fragment.

In vivo transgene construction can be used to form

5 any number of immunoglobulin transgenes which because of their size are otherwise difficult, or impossible, to make or manipulate by present technology. Thus, _in vivo_ transgene construction is useful to generate immunoglobulin transgenes which are larger than DNA fragments which may be manipulated

10 by YAC vectors (Murray and Szostak, Nature 305:189-193 (1983)). Such _in vivo_ transgene construction may be used to introduce into a non-human animal substantially the entire immunoglobulin loci from a species not consisting of the transgenic non-human animal.

15 In addition to forming genomic immunoglobulin transgenes, _in vivo_ homologous recombination may also be utilized to form "mini-locus" transgenes as described in the Examples.

In the preferred embodiments utilizing _in vivo_

20 transgene construction, each overlapping DNA fragment preferably has an overlapping substantially homologous DNA sequence between the end portion of one DNA fragment and the end portion of a second DNA fragment. Such overlapping portions of the DNA fragments preferably comprise about 500 bp

25 to about 2000 bp, most preferably 1.0 kb to 2.0 kb. Homologous recombination of overlapping DNA fragments to form transgenes _in vivo_ is further described in commonly assigned U.S. Patent Application entitled "Intracellular Generation of DNA by Homologous Recombination of DNA Fragments" filed August 29,

30 1990, under U.S.S.N. 07/574,747.

D.   Minilocus Transgenes

             As used herein, the term "immunoglobulin minilocus"
refers to a DNA sequence (which may be within a longer
sequence), usually of less than about 150 kb, typically

5   between about 25 and 100 kb, containing at least one each of
the following: a functional variable (V) gene segment, a
functional joining (J) region segment, at least one functional
constant (C) region gene segment, and--if it is a heavy chain
minilocus--a functional diversity (D) region segment, such

10   that said DNA sequence contains at least one substantial
discontinuity (e.g., a deletion, usually of at least about 2
to 5 kb, preferably 10-25 kb or more, relative to the
homologous genomic DNA sequence). A light chain minilocus
transgene will be at least 25 kb in length, typically 50 to 60

15   kb.   A heavy chain transgene will typically be about 70 to 80
kb in length, preferably at least about 60 kb with two
constant regions operably linked to switch regions.
Furthermore, the individual elements of the minilocus are
preferably in the germline configuration and capable of

20   undergoing gene rearrangement in the pre-B cell of a
transgenic animal so as to express functional antibody
molecules with diverse antigen specificities encoded entirely
by the elements of the minilocus. Further, a heavy chain
minilocus comprising at least two C_H genes and the requisite

25   switching sequences is typically capable of undergoing isotype
switching, so that functional antibody molecules of different
immunoglobulin classes will be generated. Such isotype
switching may occur in vivo in B-cells residing within the
transgenic nonhuman animal, or may occur in cultured cells of

30   the B-cell lineage which have been explanted from the
transgenic nonhuman animal.

65

In an alternate preferred embodiment, immunoglobulin heavy chain transgenes comprise one or more of each of the V_H, D, and J_H gene segments and two or more of the C_H genes. At least one of each appropriate type gene segment is

5 incorporated into the minilocus transgene. With regard to the C_H segments for the heavy chain transgene, it is preferred that the transgene contain at least one μ gene segment and at least one other constant region gene segment, more preferably a γ gene segment, and most preferably $\gamma3$ or $\gamma1$. This preference is

10 to allow for class switching between IgM and IgG forms of the encoded immunoglobulin and the production of a secretable form of high affinity non-IgM immunoglobulin. Other constant region gene segments may also be used such as those which encode for the production of IgD, IgA and IgE.

15 Those skilled in the art will also construct transgenes wherein the order of occurrence of heavy chain C_H genes will be different from the naturally-occurring spatial order found in the germline of the species serving as the donor of the C_H genes.

20 Additionally, those skilled in the art can select C_H genes from more than one individual of a species (e.g., allogeneic C_H genes) and incorporate said genes in the transgene as supernumerary C_H genes capable of undergoing isotype switching; the resultant transgenic nonhuman animal

25 may then, in some embodiments, make antibodies of various classes including all of the allotypes represented in the species from which the transgene C_H genes were obtained.

Still further, those skilled in the art can select C_H genes from different species to incorporate into the

30 transgene. Functional switch sequences are included with each C_H gene, although the switch sequences used are not necessarily those which occur naturally adjacent to the C_H gene.

Interspecies C_H gene combinations will produce a transgenic
nonhuman animal which may produce antibodies of various
classes corresponding to C_H genes from various species.
Transgenic nonhuman animals containing interspecies C_H

5   transgenes may serve as the source of B-cells for constructing
hybridomas to produce monoclonals for veterinary uses.

The heavy chain J region segments in the human
comprise six functional J segments and three pseudo genes
clustered in a 3 kb stretch of DNA. Given its relatively

10   compact size and the ability to isolate these segments
together with the μ gene and the 5' portion of the δ gene on a
single 23 kb SFiI/SpeI fragment (Sado et al., Biochem.
Biophys. Res. Comm. 154:264271 (1988), which is incorporated
herein by reference), it is preferred that all of the J region

15   gene segments be used in the mini-locus construct. Since this
fragment spans the region between the μ and δ genes, it is
likely to contain all of the 3' cis-linked regulatory elements
required for μ expression. Furthermore, because this fragment
includes the entire J region, it contains the heavy chain

20   enhancer and the μ switch region (Mills et al., Nature 306:809
(1983); Yancopoulos and Alt, Ann. Rev. Immunol. 4:339-368
(1986), which are incorporated herein by reference). It also
contains the transcription start sites which trigger VDJ
joining to form primary repertoire B-cells (Yancopoulos and

25   Alt, Cell 40:271-281 (1985), which is incorporated herein by
reference). Alternatively, a 36 kb BssHII/SpeI1 fragment,
which includes part on the D region, may be used in place of
the 23 kb SfiI/SpeI1 fragment. The use of such a fragment
increases the amount of 5' flanking sequence to facilitate

30   efficient D-to-J joining.

The human D region consists of 4 homologous 9 kb
subregions, linked in tandem (Siebenlist, et al. (1981),

Nature, 294, 631-635). Each subregion contains up to 10
individual D segments. Some of these segments have been
mapped and are shown in Fig. 4. Two different strategies are
used to generate a mini-locus D region. The first strategy

5   involves using only those D segments located in a short
contiguous stretch of DNA that includes one or two of the
repeated D subregions. A candidate is a single 15 kb fragment
that contains 12 individual D segments. This piece of DNA
consists of 2 contiguous EcoRI fragments and has been

10 completely sequenced (Ichihara, et al. (1988), EMBO J., 7,
4141-4150). Twelve D segments should be sufficient for a
primary repertoire. However, given the dispersed nature of
the D region, an alternative strategy is to ligate together
several non-contiguous D-segment containing fragments, to

15 produce a smaller piece of DNA with a greater number of
segments. Additional D-segment genes can be identified, for
example, by the presence of characteristic flanking nonamer
and heptamer sequences, supra, and by reference to the
literature.

20   At least one, and preferably more than one V gene
segment is used to construct the heavy chain minilocus
transgene. Rearranged or unrearranged V segments with or
without flanking sequences can be isolated as described in
copending applications, U.S.S.N. 07/574,748 filed August 29,

25 1990, PCT/US91/06185 filed August 28, 1991, and U.S.S.N.
07/810,279 filed December 17, 1991, each of which is
incorporated herein by reference.

            Rearranged or unrearranged V segments, D segments, J
segments, and C genes, with or without flanking sequences, can

30 be isolated as described in copending applications U.S.S.N.
07/574,748 filed August 29, 1990 and PCT/US91/06185 filed
August 28, 1991.

A minilocus light chain transgene may be similarly constructed from the human λ or κ immunoglobulin locus.

Thus, for example, an immunoglobulin heavy chain minilocus transgene construct, e.g., of about 75 kb, encoding V, D, J and constant region sequences can be formed from a plurality of DNA fragments, with each sequence being substantially homologous to human gene sequences. Preferably, the sequences are operably linked to transcription regulatory sequences and are capable of undergoing rearrangement. With two or more appropriately placed constant region sequences (e.g., μ and γ) and switch regions, switch recombination also occurs. An exemplary light chain transgene construct can be formed similarly from a plurality of DNA fragments, substantially homologous to human DNA and capable of undergoing rearrangement, as described in copending application, U.S.S.N. 07/574,748 filed August 29, 1990.

E.   Transgene Constructs Capable of Isotype Switching

Ideally, transgene constructs that are intended to undergo class switching should include all of the cis-acting sequences necessary to regulate sterile transcripts. Naturally occurring switch regions and upstream promoters and regulatory sequences (e.g., IFN-inducible elements) are preferred cis-acting sequences that are included in transgene constructs capable of isotype switching. About at least 50 basepairs, preferably about at least 200 basepairs, and more preferably at least 500 to 1000 basepairs or more of sequence immediately upstream of a switch region, preferably a human γ1 switch region, should be operably linked to a switch sequence, preferably a human γ1 switch sequence. Further, switch regions can be linked upstream of (and adjacent to) C_H genes that do not naturally occur next to the particular switch

region. For example, but not for limitation, a human γ_1 switch region may be linked upstream from a human α_2 C_H gene, or a murine γ_1 switch may be linked to a human C_H gene.

An alternative method for obtaining non-classical

5   isotype switching (e.g., δ-associated deletion) in transgenic mice involves the inclusion of the 400 bp direct repeat sequences ($\sigma\mu$ and $\epsilon\mu$) that flank the human μ gene (Yasui et al., _Eur. J. Immunol._ _19_:1399 (1989)). Homologous recombination between these two sequences deletes the μ gene

10 in IgD-only B-cells. Heavy chain transgenes can be represented by the following formulaic description:

$$(V_H)_x - (D)_y - (J_H)_z - (S_D)_m - (C_1)_n - [(T) - (S_A)_p - (C_2)]_q$$

15 where:

V$_H$ is a heavy chain variable region gene segment,

D is a heavy chain D (diversity) region gene segment,

J$_H$ is a heavy chain J (joining) region gene segment,

S$_D$ is a donor region segment capable of participating in

20   a recombination event with the S$_a$ acceptor region segments such that isotype switching occurs,

C$_1$ is a heavy chain constant region gene segment encoding
            an isotype utilized in for B cell development (e.g.,
            μ or δ),

25 T is a cis-acting transcriptional regulatory region
            segment containing at least a promoter,

S$_A$ is an acceptor region segment capable of participating
            in a recombination event with selected S$_D$ donor
            region segments, such that isotype switching occurs,

30 C$_2$ is a heavy chain constant region gene segment encoding
            an isotype other than μ (e.g., γ_1, γ_2, γ_3, γ_4, α_1, α_2,
            ϵ).

70

x, y, z, m, n, p, and q are integers. x is 1-100, n is
0-10, y is 1-50, p is 1-10, z is 1-50, q is 0-50, m
is 0-10. Typically, when the transgene is capable
of isotype switching, q must be at least 1, m is at
5   least 1, n is at least 1, and m is greater than or
equal to n.

V_H, D, J_H, S_D, C_1, T, S_A, and C_z segments may be
selected from various species, preferably mammalian species,
10   and more preferably from human and murine germline DNA.

V_H segments may be selected from various species, but
are preferably selected from V_H segments that occur naturally
in the human germline, such as V_{H251}. Typically about 2 V_H gene
segments are included, preferably about 4 V_H segments are
15   included, and most preferably at least about 10 V_H segments are
included.

At least one D segment is typically included,
although at least 10 D segments are preferably included, and
some embodiments include more than ten D segments. Some
20   preferred embodiments include human D segments.

Typically at least one J_H segment is incorporated in
the transgene, although it is preferable to include about six
J_H segments, and some preferred embodiments include more than
about six J_H segments. Some preferred embodiments include
25   human J_H segments, and further preferred embodiments include
six human J_H segments and no nonhuman J_H segments.

S_D segments are donor regions capable of
participating in recombination events with the S_A segment of
the transgene. For classical isotype switching, S_D and S_A are
30   switch regions such as S_μ, $S_{\gamma1}$, $S_{\gamma2}$, $S_{\gamma3}$, $S_{\gamma4}$, S_α, $S_{\alpha2}$, and S_ϵ.
Preferably the switch regions are murine or human, more
preferably S_D is a human or murine S_μ and S_A is a human or

murine $S_{\gamma 1}$. For nonclassical isotype switching (δ-associated deletion), S_D and S_A are preferably the 400 basepair direct repeat sequences that flank the human μ gene.

C_1 segments are typically μ or δ genes, preferably a
5 μ gene, and more preferably a human or murine μ gene.

T segments typically include S' flanking sequences that are adjacent to naturally occurring (i.e., germline) switch regions. T segments typically at least about at least 50 nucleotides in length, preferably about at least 200
10 nucleotides in length, and more preferably at least 500-1000 nucleotides in length. Preferably T segments are 5' flanking sequences that occur immediately upstream of human or murine switch regions in a germline configuration. It is also evident to those of skill in the art that T segments may
15 comprise cis-acting transcriptional regulatory sequences that do not occur naturally in an animal germline (e.g., viral enhancers and promoters such as those found in SV40, adenovirus, and other viruses that infect eukaryotic cells).

C_2 segments are typically a γ_1, γ_2, γ_3, γ_4, α_1, α_2, or
20 ϵ C_H gene, preferably a human c_H gene of these isotypes, and more preferably a human γ_1 or γ_3 gene. Murine γ_{2a} and γ_{2b} may also be used, as may downstream (i.e., switched) isotype genes form various species. Where the heavy chain transgene contains an immunoglobulin heavy chain minilocus, the total length of the
25 transgene will be typically 150 kilo basepairs or less.

In general, the transgene will be other than a native heavy chain Ig locus. Thus, for example, deletion of unnecessary regions or substitutions with corresponding regions from other species will be present.

30

F.   Methods for Determining Functional
     Isotype Switching in Ig Transgenes

The occurrence of isotype switching in a transgenic nonhuman animal may be identified by any method known to those in the art. Preferred embodiments include the following, employed either singly or in combination:

5   1. detection of mRNA transcripts that contain a sequence homologous to at least one transgene downstream C_H gene other than δ and an adjacent sequence homologous to a transgene V_H-D_H-J_H rearranged gene; such detection may be by Northern hybridization, S_1 nuclease protection assays, PCR

10   amplification, cDNA cloning, or other methods;

       2. detection in the serum of the transgenic animal, or in supernatants of cultures of hybridoma cells made from B-cells of the transgenic animal, of immunoglobulin proteins encoded by downstream C_H genes, where such proteins can also be shown

15   by immunochemical methods to comprise a functional variable region;

       3. detection, in DNA from B-cells of the transgenic animal or in genomic DNA from hybridoma cells, of DNA rearrangements consistent with the occurrence of isotype

20   switching in the transgene, such detection may be accomplished by Southern blot hybridization, PCR amplification, genomic cloning, or other method; or

       4. identification of other indicia of isotype switching, such as production of sterile transcripts, production of

25   characteristic enzymes involved in switching (e.g., "switch recombinase"), or other manifestations that may be detected, measured, or observed by contemporary techniques.

       Because each transgenic line may represent a different site of integration of the transgene, and a

30   potentially different tandem array of transgene inserts, and because each different configuration of transgene and flanking DNA sequences can affect gene expression, it is preferable to

identify and use lines of mice that express high levels of human immunoglobulins, particularly of the IgG isotype, and contain the least number of copies of the transgene. Single copy transgenics minimize the potential problem of incomplete allelic expression. Transgenes are typically integrated into host chromosomal DNA, most usually into germline DNA and propagated by subsequent breeding of germline transgenic breeding stock animals. However, other vectors and transgenic methods known in the present art or subsequently developed may be substituted as appropriate and as desired by a practitioner.

Trans-switching to endogenous nonhuman heavy chain constant region genes can occur and produce chimeric heavy chains and antibodies comprising such chimeric human/mouse heavy chains. Such chimeric antibodies may be desired for certain uses described herein or may be undesirable.

G. Functional Disruption of
Endogenous Immunoglobulin Loci

The expression of successfully rearranged immunoglobulin heavy and light transgenes is expected to have a dominant effect by suppressing the rearrangement of the endogenous immunoglobulin genes in the transgenic nonhuman animal. However, another way to generate a nonhuman that is devoid of endogenous antibodies is by mutating the endogenous immunoglobulin loci. Using embryonic stem cell technology and homologous recombination, the endogenous immunoglobulin repertoire can be readily eliminated. The following describes the functional description of the mouse immunoglobulin loci. The vectors and methods disclosed, however, can be readily adapted for use in other non-human animals.

Briefly, this technology involves the inactivation of a gene, by homologous recombination, in a pluripotent cell

line that is capable of differentiating into germ cell tissue.
A DNA construct that contains an altered, copy of a mouse
immunoglobulin gene is introduced into the nuclei of embryonic
stem cells. In a portion of the cells, the introduced DNA

5   recombines with the endogenous copy of the mouse gene,
replacing it with the altered copy. Cells containing the
newly engineered genetic lesion are injected into a host mouse
embryo, which is reimplanted into a recipient female. Some of
these embryos develop into chimeric mice that possess germ

10   cells entirely derived from the mutant cell line. Therefore,
by breeding the chimeric mice it is possible to obtain a new
line of mice containing the introduced genetic lesion
(reviewed by Capecchi (1989), Science, 244, 1288-1292).

Because the mouse λ locus contributes to only 5% of

15   the immunoglobulins, inactivation of the heavy chain and/or
κ-light chain loci is sufficient. There are three ways to
disrupt each of these loci, deletion of the J region, deletion
of the J-C intron enhancer, and disruption of constant region
coding sequences by the introduction of a stop codon. The

20   last option is the most straightforward, in terms of DNA
construct design. Elimination of the μ gene disrupts B-cell
maturation thereby preventing class switching to any of the
functional heavy chain segments. The strategy for knocking
out these loci is outlined below.

25   To disrupt the mouse μ and κ genes, targeting
vectors are used based on the design employed by Jaenisch and
co-workers (Zijlstra, et al. (1989), Nature, 342, 435-438) for
the successful disruption of the mouse β2-microglobulin gene.
The neomycin resistance gene (neo), from the plasmid pMCIneo

30   is inserted into the coding region of the target gene. The
pMCIneo insert uses a hybrid viral promoter/enhancer sequence
to drive neo expression. This promoter is active in embryonic

stem cells. Therefore, neo can be used as a selectable marker for integration of the knock-out construct. The HSV thymidine kinase (tk) gene is added to the end of the construct as a negative selection marker against random insertion events

5   (Zijlstra, et al., supra.).

A preferred strategy for disrupting the heavy chain locus is the elimination of the J region. This region is fairly compact in the mouse, spanning only 1.3 kb. To construct a gene targeting vector, a 15 kb KpnI fragment

10 containing all of the secreted A constant region exons from mouse genomic library is isolated. The 1.3 kb J region is replaced with the 1.1 kb insert from pMCIneo. The HSV tk gene is then added to the 5' end of the KpnI fragment. Correct integration of this construct, via homologous recombination,

15 will result in the replacement of the mouse J_H region with the neo gene. Recombinants are screened by PCR, using a primer based on the neo gene and a primer homologous to mouse sequences 5' of the KpnI site in the D region.

Alternatively, the heavy-chain locus is knocked out

20 by disrupting the coding region of the μ gene. This approach involves the same 15 kb KpnI fragment used in the previous approach. The 1.1 kb insert from pMCIneo is inserted at a unique BamHI site in exon II, and the HSV tk gene added to the 3' KpnI end. Double crossover events on either side of the

25 neo insert, that eliminate the tk gene, are then selected for. These are detected from pools of selected clones by PCR amplification. One of the PCR primers is derived from neo sequences and the other from mouse sequences outside of the targeting vector. The functional disruption of the mouse

30 immunoglobulin loci is presented in the Examples.

G.   Suppressing Expression of

Endogenous Immunoglobulin Loci

        In addition to functional disruption of endogenous
Ig loci, an alternative method for preventing the expression
of an endogenous Ig locus is suppression. Suppression of
5   endogenous Ig genes may be accomplished with antisense RNA
produced from one or more integrated transgenes, by antisense
oligonucleotides, and/or by administration of antisera
specific for one or more endogenous Ig chains.

10   Antisense Polynucleotides

        Antisense RNA transgenes can be employed to
partially or totally knock-out expression of specific genes
(Pepin et al. (1991) Nature 355: 725; Helene., C. and Toulme,
J. (1990) Biochimica Biophys. Acta 1049: 99; Stout, J. and
15 Caskey, T. (1990) Somat. Cell Mol. Genet. 16: 369; Munir et
al. (1990) Somat. Cell Mol. Genet. 16: 383, each of which is
incorporated herein by reference).

        "Antisense polynucleotides" are polynucleotides
that: (1) are complementary to all or part of a reference
20 sequence, such as a sequence of an endogenous Ig C_H or C_L
region, and (2) which specifically hybridize to a
complementary target sequence, such as a chromosomal gene
locus or a Ig mRNA. Such complementary antisense
polynucleotides may include nucleotide substitutions,
25 additions, deletions, or transpositions, so long as specific
hybridization to the relevant target sequence is retained as a
functional property of the polynucleotide. Complementary
antisense polynucleotides include soluble antisense RNA or DNA
oligonucleotides which can hybridize specifically to
30 individual mRNA species and prevent transcription and/or RNA
processing of the mRNA species and/or translation of the
encoded polypeptide (Ching et al., Proc. Natl. Acad. Sci.

U.S.A. 86:10006-10010 (1989); Broder et al., Ann. Int. Med. 113:604-618 (1990); Loreau et al., FEBS Letters 274:53-56 (1990); Holcenberg et al., WO91/11535; U.S.S.N. 07/530,165 ("New human CRIPTO gene"); WO91/09865; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). An antisense sequence is a polynucleotide sequence that is complementary to at least one immunoglobulin gene sequence of at least about 15 contiguous nucleotides in length, typically at least 20 to 30 nucleotides in length, and preferably more than about 30 nucleotides in length. However, in some embodiments, antisense sequences may have substitutions, additions, or deletions as compared to the complementary immunoglobulin gene sequence, so long as specific hybridization is retained as a property of the antisense polynucleotide. Generally, an antisense sequence is complementary to an endogenous immunoglobulin gene sequence that encodes, or has the potential to encode after DNA rearrangement, an immunoglobulin chain. In some cases, sense sequences corresponding to an immunoglobulin gene sequence may function to suppress expression, particularly by interfering with transcription.

The antisense polynucleotides therefore inhibit production of the encoded polypeptide(s). In this regard, antisense polynucleotides that inhibit transcription and/or translation of one or more endogenous Ig loci can alter the capacity and/or specificity of a non-human animal to produce immunoglobulin chains encoded by endogenous Ig loci.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell, such as a transgenic pluripotent hematopoietic stem cell used to reconstitute all or part of the hematopoietic stem cell population of an individual, or a

transgenic nonhuman animal. Alternatively, the antisense

polynucleotides may comprise soluble oligonucleotides that are

administered to the external milieu, either in culture medium

in *vitro* or in the circulatory system or interstitial fluid *in*

5   *vivo*. Soluble antisense polynucleotides present in the

external milieu have been shown to gain access to the

cytoplasm and inhibit translation of specific mRNA species. In

some embodiments the antisense polynucleotides comprise

methylphosphonate moieties, alternatively phosphorothiolates

10   or O-methylribonucleotides may be used, and chimeric

oligonucleotides may also be used (Dagle et al. (1990) *Nucleic*

Acids Res. *18*: 4751). For some applications, antisense

oligonucleotides may comprise polyamide nucleic acids (Nielsen

et al. (1991) *Science* *254*: 1497). For general methods

15   relating to antisense polynucleotides, see *Antisense RNA and*

DNA, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory,

Cold Spring Harbor, NY).

        Antisense polynucleotides complementary to one or

more sequences are employed to inhibit transcription, RNA

20   processing, and/or translation of the cognate mRNA species and

thereby effect a reduction in the amount of the respective

encoded polypeptide. Such antisense polynucleotides can

provide a therapeutic function by inhibiting the formation of

one or more endogenous Ig chains *in vivo*.

25   Whether as soluble antisense oligonucleotides or as

antisense RNA transcribed from an antisense transgene, the

antisense polynucleotides of this invention are selected so as

to hybridize preferentially to endogenous Ig sequences at

physiological conditions *in vivo*. Most typically, the

30   selected antisense polynucleotides will not appreciably

hybridize to heterologous Ig sequences encoded by a heavy or

light chain transgene of the invention (i.e., the antisense

oligonucleotides will not inhibit transgene Ig expression by more than about 25 to 35 percent).

Antiserum Suppression

Partial or complete suppression of endogenous Ig chain expression can be produced by injecting mice with antisera against one or more endogenous Ig chains (Weiss et al. (1984) _Proc. Natl. Acad. Sci. (U.S.A.)_ **81** 211, which is incorporated herein by reference). Antisera are selected so as to react specifically with one or more endogenous (e.g., murine) Ig chains but to have minimal or no cross-reactivity with heterologous Ig chains encoded by an Ig transgene of the invention. Thus, administration of selected antisera according to a schedule as typified by that of Weiss et al. _op.cit._ will suppress endogenous Ig chain expression but permits expression of heterologous Ig chain(s) encoded by a transgene of the present invention. Suitable antibody sources for antibody comprise:

(1) monoclonal antibodies, such as a monoclonal antibody that specifically binds to a murine μ, γ, κ, or λ chains but does not react with the human immunoglobulin chain(s) encoded by a human Ig transgene of the invention;

(2) mixtures of such monoclonal antibodies, so that the mixture binds with multiple epitopes on a single species of endogenous Ig chain, with multiple endogenous Ig chains (e.g., murine μ and murine γ, or with multiple epitopes and multiple chains or endogenous immunoglobulins;

(3) polyclonal antiserum or mixtures thereof, typically such antiserum/antisera is monospecific for binding to a single species of endogenous Ig chain (e.g., murine μ, murine γ, murine κ, murine λ) or to multiple species of endogenous Ig chain, and most preferably such antisera

possesses negligible binding to human immunoglobulin chains
encoded by a transgene of the invention; and/or

      (4) a mixture of polyclonal antiserum and monoclonal
antibodies binding to a single or multiple species of

5 endogenous Ig chain, and most preferably possessing negligible
binding to human immunoglobulin chains encoded by a transgene
of the invention. Generally, polyclonal antibodies are
preferred, and such substantially monospecific polyclonal
antibodies can be advantageously produced from an antiserum

10 raised against human immunoglobulin(s) by pre-adsorption with
antibodies derived from the nonhuman animal species (e.g.,
murine) and/or, for example, by affinity chromatography of the
antiserum or purified fraction thereof on an affinity resin
containing immobilized human Ig (wherein the bound fraction is

15 enriched for the desired anti-human Ig in the antiserum; the
bound fraction is typically eluted with conditions of low pH
or a chaotropic salt solution).

      Cell separation and/or complement fixation can be
employed to provide the enhancement of antibody-directed cell

20 depletion of lymphocytes expressing endogenous (e.g., murine)
immunoglobulin chains. In one embodiment, for example,
antibodies are employed for _ex vivo_ depletion of murine Ig-
expressing explanted hematopoietic cells and/or B-lineage
lymphocytes obtained from a transgenic mouse harboring a human

25 Ig transgene. Thus, hematopoietic cells and/or B-lineage
lymphocytes are explanted from a transgenic nonhuman animal
harboring a human Ig transgene (preferably harboring both a
human heavy chain transgene and a human light chain transgene)
and the explanted cells are incubated with an antibody (or

30 antibodies) which (1) binds to an endogenous immunoglobulin
(e.g., murine μ and/or κ) and (2) lacks substantial binding to
human immunoglobulin chains encoded by the transgene(s). Such

antibodies are referred to as "suppression antibodies" for

clarity. The explanted cell population is selectively

depleted of cells which bind to the suppression antibody(ies);

such depletion can be accomplished by various methods, such as

5   (1) physical separation to remove suppression antibody-bound

cells from unbound cells (e.g., the suppression antibodies may

be bound to a solid support or magnetic bead to immobilize and

remove cells binding to the suppression antibody), (2)

antibody-dependent cell killing of cells bound by the

10   suppression antibody (e.g., by ADCC, by complement fixation,

or by a toxin linked to the suppression antibody), and (3)

clonal anergy induced by the suppression antibody, and the

like.

           Frequently, antibodies used for antibody suppression

15   of endogenous Ig chain production will be capable of fixing

complement. It is frequently preferable that such antibodies

may be selected so as to react well with a convenient

complement source for ex vivo/in vitro depletion, such as

rabbit or guinea pig complement. For in vivo depletion, it is

20   generally preferred that the suppressor antibodies possess

effector functions in the nonhuman transgenic animal species;

thus, a suppression antibody comprising murine effector

functions (e.g., ADCC and complement fixation) generally would

be preferred for use in transgenic mice.

25   In one variation, a suppression antibody that

specifically binds to a predetermined endogenous

immunoglobulin chain is used for ex vivo/in vitro depletion

of lymphocytes expressing an endogenous immunoglobulin. A

cellular explant (e.g., lymphocyte sample) from a transgenic

30   nonhuman animal harboring a human immunoglobulin transgene is

contacted with a suppression antibody and cells specifically

binding to the suppression antibody are depleted (e.g., by

immobilization, complement fixation, and the like), thus
generating a cell subpopulation depleted in cells expressing
endogenous (nonhuman) immunoglobulins (e.g., lymphocytes
expressing murine Ig). The resultant depleted lymphocyte

5   population (T cells, human Ig-positive B-cells, etc.) can be
transferred into a immunocompatible (i.e., MHC-compatible)
nonhuman animal of the same species and which is substantially
incapable of producing endogenous antibody (e.g., SCID mice,
RAG-1 or RAG-2 knockout mice). The reconstituted animal

10   (mouse) can then be immunized with an antigen (or reimmunized
with an antigen used to immunize the donor animal from which
the explant was obtained) to obtain high-affinity (affinity
matured) antibodies and B-cells producing such antibodies.
Such B-cells may be used to generate hybridomas by

15   conventional cell fusion and screened. Antibody suppression
can be used in combination with other endogenous Ig
inactivation/suppression methods (e.g., J_H knockout, C_H
knockout, D-region ablation, antisense suppression,
compensated frameshift inactivation).

20

Complete Endogenous Ig Locus Inactivation

In certain embodiments, it is desirable to effect
complete inactivation of the endogenous Ig loci so that hybrid
immunoglobulin chains comprising a human variable region and a

25   non-human (e.g., murine) constant region cannot be formed
(e.g., by trans-switching between the transgene and endogenous
Ig sequences). Knockout mice bearing endogenous heavy chain
alleles with are functionally disrupted in the J_H region only
frequently exhibit trans-switching, typically wherein a

30   rearranged human variable region (VDJ) encoded by a transgene
is expressed as a fusion protein linked to an endogenous
murine constant region, although other trans-switched

junctions are possible. To overcome this potential problem,
it is generally desirable to completely inactivate the
endogenous heavy chain locus by any of various methods,
including but not limited to the following: (1) functionally

5   disrupting and/or deleting by homologous recombination at
least one and preferably all of the endogenous heavy chain
constant region genes, (2) mutating at least one and
preferably all of the endogenous heavy chain constant region
genes to encode a termination codon (or frameshift) to produce

10 a truncated or frameshifted product (if trans-switched), and
other methods and strategies apparent to those of skill in the
art. Deletion of a substantial portion or all of the heavy
chain constant region genes and/or D-region genes may be
accomplished by various methods, including sequential deletion

15 by homologous recombination targeting vectors, especially of
the "hit-and-run" type and the like. Similarly, functional
disruption and/or deletion of at least one endogenous light
chain locus (e.g., κ) to ablate endogenous light chain
constant region genes is often preferable.

20   Frequently, it is desirable to employ a frameshifted
transgene wherein the heterologous transgene comprises a
frameshift in the J segment(s) and a compensating frameshift
(i.e., to regenerate the original reading frame) in the
initial region (i.e., amino-terminal coding portion) of one or

25 more (preferably all) of the transgene constant region genes.
Trans-switching to an endogenous IgH locus constant gene
(which does not comprise a compensating frameshift) will
result in a truncated or missense product that results in the
trans-switched B cell being deleted or non-selected, thus

30 suppressing the trans-switched phenotype.
             Antisense suppression and antibody suppression may
also be used to effect a substantially complete functional

inactivation of endogenous Ig gene product expression (e.g.,
murine heavy and light chain sequences) and/or trans-switched
antibodies (e.g., human variable/murine constant chimeric
antibodies).

5   Various combinations of the inactivation and
suppression strategies may be used to effect essentially total
suppression of endogenous (e.g., murine) Ig chain expression.

                        <u>Trans-Switching</u>

10   In some variations, it may be desirable to produce a
trans-switched immunoglobulin. For example, such trans-
switched heavy chains can be chimeric (i.e., a non-murine
(human) variable region and a murine constant region).
Antibodies comprising such chimeric trans-switched
15 immunoglobulins can be used for a variety of applications
where it is desirable to have a non-human (e.g., murine)
constant region (e.g., for retention of effector functions in
the host, for the presence of murine immunological
determinants such as for binding of a secondary antibody which
20 does not bind human constant regions). For one example, a
human variable region repertoire may possess advantages as
compared to the murine variable region repertoire with respect
to certain antigens. Presumably the human V_H, D, J_H, V_L, and J_L
genes have been selected for during evolution for their
25 ability to encode immunoglobulins that bind certain
evolutionarily important antigens; antigens which provided
evolutionary selective pressure for the murine repertoire can
be distinct from those antigens which provided evolutionary
pressure to shape the human repertoire. Other repertoire
30 adavantages may exist, making the human variable region
repertoire advantageous when combined with a murine constant
region (e.g., a trans-switched murine) isotype. The presence

of a murine constant region can afford advantages over a human
constant region. For example, a murine γ constant region
linked to a human variable region by trans-switching may
provide an antibody which possesses murine effector functions
5   (e.g., ADCC, murine complement fixation) so that such a
chimeric antibody (preferably monoclonal) which is reactive
with a predetermined antigen (e.g., human IL-2 receptor) may
be tested in a mouse disease model, such as a mouse model of
graft-versus-host disease wherein the T lymphocytes in the
10 mouse express a functional human IL-2 receptor. Subsequently,
the human variable region encoding sequence may be isolated
(e.g., by PCR amplification or cDNA cloning from the source
(hybridoma clone)) and spliced to a sequence encoding a
desired human constant region to encode a human sequence
15 antibody more suitable for human therapeutic uses where
immunogenicity is preferably minimized. The polynucleotide(s)
having the resultant fully human encoding sequence(s) can be
expressed in a host cell (e.g., from an expression vector in a
mammalian cell) and purified for pharmaceutical formulation.
20 For some applications, the chimeric antibodies may be used
directly without replacing the murine constant region with a
human constant region. Other variations and uses of trans-
switched chimeric antibodies will be evident to those of skill
in the art.

25   The present invention provides transgenic nonhuman
animals containing B lymphocytes which express chimeric
antibodies, generally resulting from trans-switching between a
human heavy chain transgene and an endogenous murine heavy
chain constant region gene. Such chimeric antibodies comprise
30 a human sequence variable region and a murine constant region,
generally a murine switched (i.e., non-μ, non-δ) isotype. The
transgenic nonhuman animals capable of making chimeric

antibodies to a predetermined antigen are usually also competent to make fully human sequence antibodies if both human heavy chain and human light chain transgenes encoding human variable and human constant region genes are integrated.

5 Most typically, the animal is homozygous for a functionally disrupted heavy chain locus and/or light chain locus but retains one or more endogenous heavy chain constant region gene(s) capable of trans-switching (e.g., γ, α, e) and frequently retains a cis-linked enhancer. Such a mouse is

10 immunized with a predetermined antigen, usually in combination with an adjuvant, and an immune response comprising a detectable amount of chimeric antibodies comprising heavy chains composed of human sequence variable regions linked to murine constant region sequences is produced. Typically, the

15 serum of such an immunized animal can comprise such chimeric antibodies at concentrations of about at least 1 μg/ml, often about at least 10 μg/ml, frequently at least 30 μg/ml, and up to 50 to 100 μg/ml or more. The antiserum containing antibodies comprising chimeric human variable/mouse constant

20 region heavy chains typically also comprises antibodies which comprise human variable/human constant region (complete human sequence) heavy chains. Chimeric trans-switched antibodies usually comprise (1) a chimeric heavy chain composed of a human variable region and a murine constant region (typically

25 a murine gamma) and (2) a human transgene-encoded light chain (typically kappa) or a murine light chain (typically lambda in a kappa knockout background). Such chimeric trans-switched antibodies generally bind to a predetermined antigen (e.g., the immunogen) with an affinity of about at least 1 x 10^7 M^{-1},

30 preferably with an affinity of about at least 5 x 10^7 M^{-1}, more preferably with an affinity of at least 1 x 10^8 M^{-1} to 1 x 10^9 M^{-1} or more. Frequently, the predetermined antigen is a human

protein, such as for example a human cell surface antigen (e.g., CD4, CD8, IL-8, IL-2 receptor, EGF receptor, PDGF receptor), other human biological macromolecule (e.g., thrombomodulin, protein C, carbohydrate antigen, sialyl Lewis

5 antigen, L-selectin), or nonhuman disease associated macromolecule (e.g., bacterial LPS, virion capsid protein or envelope glycoprotein) and the like.

The invention provides transgenic nonhuman animals comprising a genome comprising: (1) a homozygous functionally
10 disrupted endogenous heavy chain locus comprising at least one murine constant region gene capable of trans-switching (e.g., in cis linkage to a functional switch recombination sequence and typically to a functional enhancer), (2) a human heavy chain transgene capable of rearranging to encode end express a
15 functional human heavy chain variable region and capable of trans-switching (e.g., having a cis-linked RSS); optionally further comprising (3) a human light chain (e.g., kappa) transgene capable of rearranging to encode a functional human light chain variable region and expressing a human sequence
20 light chain; optionally further comprising (4) a homozygous functionally disrupted endogenous light chain locus (κ, preferably κ and λ); and optionally further comprising (5) a serum comprising an antibody comprising a chimeric heavy chain composed of a human sequence variable region encoded by a
25 human transgene and a murine constant region sequence encoded by an endogenous murine heavy chain constant region gene (e.g., γ1, γ2a, γ2b, γ3).

Such transgenic mice may further comprise a serum comprising chimeric antibodies which bind a predetermined
30 human antigen (e.g., CD4, CD8, CEA) with an affinity of about at least 1×10^4 M^{-1}, preferably with an affinity of about at least 5×10^4 M^{-1}, more preferably with an affinity of at least

1×10^7 M^{-1} to 1×10^9 M^{-1} or more. Frequently, hybridomas can be made wherein the monoclonal antibodies produced thereby have an affinity of at least 8×10^7 M^{-1}. Chimeric antibodies comprising a heavy chain composed of a murine constant region

5 and a human variable region, often capable of binding to a nonhuman antigen, may also be present in the serum or as an antibody secreted from a hybridoma.

In some variations, it is desirable to generate transgenic mice which have inactivated endogenous mouse heavy

10 chain loci which retain intact heavy chain constant region genes, and which have a human heavy chain transgene capable of trans-switching, and optionally also have a human light chain transgene, optionally with one or more inactivated endogenous mouse light chain loci. Such mice may advantageously produce

15 B cells capable of alternatively expressing antibodies comprising fully human heavy chains and antibodies comprising chimeric (human variable/mouse constant) heavy chains, by trans-switching. The serum of said mice would contain antibodies comprising fully human heavy chains and antibodies

20 comprising chimeric (human variable/mouse constant) heavy chains, preferably in combination with fully human light chains. Hybridomas can be generated from the B cells of said mice.

Generally, such chimeric antibodies can be generated

25 by trans-switching, wherein a human transgene encoding a human variable region (encoded by productive V-D-J rearrangement *in vivo*) and a human constant region, typically human μ, undergoes switch recombination with a non-transgene immunoglobulin constant gene switch sequence (RSS) thereby

30 operably linking the transgene-encoded human variable region with a heavy chain constant region which is not encoded by said transgene, typically an endogenous murine immunoglobulin

heavy chain constant region or a heterologous (e.g., human)
heavy chain constant region encoded on a second transgene.
Whereas cis-switching refers to isotype-switching by
recombination of RSS elements *within* a transgene, trans-

5   switching involves recombination between a transgene RSS and
an RSS element outside the transgene, often on a different
chromosome than the chromosome which harbors the transgene.

Trans-switching generally occurs between an RSS of
an expressed transgene heavy chain constant region gene and

10   either an RSS of an endogenous murine constant region gene (of
a non-μ isotype, typically γ) or an RSS of a human constant
region gene contained on a second transgene, often integrated
on a separate chromosome.

When trans-switching occurs between an RSS of a

15   first, expressed transgene heavy chain constant region gene
(e.g., μ) and an RSS of a human heavy chain constant region
gene contained on a second transgene, a non-chimeric antibody
having a substantially fully human sequence is produced. For
example and not limitation, a polynucleotide encoding a human

20   heavy chain constant region (e.g., $\gamma 1$) and an operably linked
RSS (e.g., a $\gamma 1$ RSS) can be introduced (e.g., transfected)
into a population of hybridoma cells generated from a
transgenic mouse B-cell (or B cell population) expressing an
antibody comprising a transgene-encoded human μ chain. The

25   resultant hybridoma cells can be selected for the presence of
the introduced polynucleotide and/or for the expression of
trans-switched antibody comprising a heavy chain having the
variable region (idiotype/antigen reactivity) of the human μ
chain and having the constant region encoded by the introduced

30   polynucleotide sequence (e.g., human $\gamma 1$). Trans-switch
recombination between the RSS of the transgene-encoded human μ
chain and the RSS of the introduced polynucleotide encoding a

downstream isotype (e.g., γ1) thereby can generate a trans-switched antibody.

The invention also provides a method for producing such chimeric trans-switched antibodies comprising the step of immunizing with a predetermined antigen a transgenic mouse comprising a genome comprising: (1) a homozygous functionally disrupted endogenous heavy chain locus comprising at least one murine constant region gene capable of trans-switching (e.g., γ2a, γ2b, γ1, γ3), (2) a human heavy chain transgene capable of rearranging to encode a functional human heavy chain variable region and expressing a human sequence heavy chain and capable of undergoing isotype switching (and/or trans-switching), and optionally further comprising (3) a human light chain (e.g., kappa) transgene capable of rearranging to encode a functional human light (e.g., kappa) chain variable region and expressing a human sequence light chain, and optionally further comprising (4) a homozygous functionally disrupted endogenous light chain locus (typically κ, preferably both κ and λ), and optionally further comprising (5) a serum comprising an antibody comprising a chimeric heavy chain composed of a human sequence variable region encoded by a human transgene and a murine constant region sequence encoded by an endogenous murine heavy chain constant region gene (e.g., γ1, γ2a, γ2b, γ3).

Affinity Tagging: Selecting for Switched Isotypes

Advantageously, trans-switching (and cis-switching) is associated with the process of somatic mutation. Somatic mutation expands the range of antibody affinities encoded by clonal progeny of a B-cell. For example, antibodies produced by hybridoma cells which have undergone switching (trans- or cis-) represent a broader range of antigen-binding affinities

than is present in hybridoma cells which have not undergone
switching. Thus, a hybridoma cell population (typically
clonal) which expresses a first antibody comprising a heavy
chain comprising a first human heavy chain variable region in
5 polypeptide linkage to a first human heavy chain constant
region (e.g., μ) can be screened for hybridoma cell clonal
variants which express an antibody comprising a heavy chain
containing said first human heavy chain variable region in
polypeptide linkage to a second heavy chain constant region
10 (e.g., a human γ, α, or ϵ constant region). Such clonal
variants can be produced by natural clonal variation producing
cis-switching in vitro, by induction of class switching
(trans- or cis-) as through the administration of agents that
promote isotype switching, such as T-cell-derived lymphokines
15 (e.g., IL-4 and IFN$_\gamma$), by introduction of a polynucleotide
comprising a functional RSS and a heterologous (e.g. human)
heavy chain constant region gene to serve as a substrate for
trans-switching, or by a combination of the above, and the
like. Often, polynucleotides containing a human downstream
20 isotype constant region (e.g., γ1, γ3, and the like) with an
operably linked RSS will also be introduced into hybridoma
cells to promote isotype switching via the trans-switch
mechanism.

Class switching and affinity maturation take place
25 within the same population of B cells derived from transgenic
animals of the present invention. Therefore, identification
of class-switched B cells (or hybridomas derived therefrom)
can be used as a screening step for obtaining high affinity
monoclonal antibodies. A variety of approaches can be
30 employed to facilitate class switching events such as cis-
switching (intratransgene switching), trans-switching, or
both. For example, a single continuous human genomic fragment

comprising both μ and γ constant region genes with the associated RSS elements and switch regulatory elements (e.g., sterile transcript promoter) can be used as a transgene. However, some portions of the desired single contiguous human

5   genomic fragment can be difficult to clone efficiently, such as due to instability problems when replicated in a cloning host or the like; in particular, the region between δ and $\gamma3$ can prove difficult to clone efficiently, especially as a contiguous fragment comprising the μ gene, $\gamma3$ gene, a V gene,

10 D gene segments, and J gene segments.

        Also for example, a discontinuous human transgene (minigene) composed of a human μ gene, human $\gamma3$ gene, a human V gene(s), human D gene segments, and human J gene segments, with one or more deletions of an intervening (intronic) or

15 otherwise nonessential sequence (e.g., one or more V, D, and/or J segment and/or one or more non-μ constant region gene(s)). Such minigenes have several advantages as compared to isolating a single contiguous segment of genomic DNA spanning all of the essential elements for efficient

20 immunoglobulin expression and switching. For example, such a minigene avoids the necessity of isolating large pieces of DNA which may contain sequences which are difficult to clone (e.g., unstable sequences, poison sequences, and the like). Moreover, miniloci comprising elements necessary for isotype

25 switching (e.g., human γ sterile transcript promoter) for producing cis- or trans-switching, can advantageously undergo somatic mutation and class switching in vivo. As many eukaryotic DNA sequences can prove difficult to clone, omitting non-essential sequences can prove advantageous.

30   In a variation, hybridoma clones producing antibodies having high binding affinity (e.g., at least 1×10^7 M^{-1}, preferably at least 1×10^8 M^{-1}, more preferably at least 1

x 10^9 M^{-1} or greater) are obtained by selecting, from a pool of
hybridoma cells derived from B cells of transgenic mice
harboring a human heavy chain transgene capable of isotype
switching (see, supra) and substantially lacking endogenous

5   murine heavy chain loci capable of undergoing productive (in-
frame) V-D-J rearrangement, hybridomas which express an
antibody comprising a heavy chain comprising a human sequence
heavy chain variable region in polypeptide linkage to a human
(or mouse) non-μ heavy chain constant region; said antibodys

10   are termed "switched antibodies" as they comprise a "switched
heavy chain" which is produced as a consequence of cis-
switching and/or trans-switching in vivo or in cell culture.
Hybridomas producing switched antibodies generally have
undergone the process of somatic mutation, and a pool of said

15   hybridomas will generally have a broader range of antigen
binding affinities from which hybridoma clones secreting high
affinity antibodies can be selected. Typically, hybridomas
secreting a human sequence antibody having substantial binding
affinity (greater than 1 x 10^7 M^{-1} to 1 x 10^8 M^{-1}) for a

20   predetermined antigen and wherein said human sequence antibody
comprises human immunoglobulin variable region(s) can be
selected by a method comprising a two-step process. One step
is to identify and isolate hybridoma cells which secrete
immunoglobulins which comprise a switched heavy chain (e.g.,

25   by binding hybridoma cells to an immobilized immunoglobulin
which specifically binds a switched heavy chain and does not
substantially bind to an unswitched isotype, e.g., μ). The
other step is to identify hybridoma cells which bind to the
predetermined antigen with substantial binding affinity (e.g.,

30   by ELISA of hybridoma clone supernatants, FACS analysis using
labeled antigen, and the like). Typically, selection of
hybridomas which secrete switched antibodies is performed

prior to identifying hybridoma cells which bind predetermined antigen. Hybridoma cells which express switched antibodies that have substantial binding affinity for the predetermined antigen are isolated and cultured under suitable growth

5 conditions known in the art, typically as individual selected clones. Optionally, the method comprises the step of culturing said selected clones under conditions suitable for expression of monocloanl antibodies; said monoclonal antibodies are collected and can be administered for therapeutic,

10 prophylactic, and/or diagnostic purposes.

Often, the selected hybridoma clones can serve as a source of DNA or RNA for isolating immunoglobulin sequences which encode immunoglobulins (e.g. a variable region) that bind to (or confer binding to) the predetermined antigen.

15 Subsequently, the human variable region encoding sequence may be isolated (e.g., by PCR amplification or cDNA cloning from the source (hybridoma clone)) and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic uses

20 where immunogenicity is preferably minimized. The polynucleotide(s) having the resultant fully human encoding sequence(s) can be expressed in a host cell (e.g., from an expression vector in a mammalian cell) and purified for pharmaceutical formulation.

25

Xenoenhancers

A heterologous transgene capable of encoding a human immunoglobulin (e.g., a heavy chain) advantageously comprises a cis-linked enhancer which is not derived from the mouse

30 genome, and/or which is not naturally associated in cis with the exons of the heterologous transgene. For example, a human κ transgene (e.g., a κ minilocus) can advantageously comprise

a human Vκ gene, a human Jκ gene, a human Cκ gene, and a

xenoenhancer, typically said xenoenhancer comprises a human

heavy chain intronic enhancer and/or a murine heavy chain

intronic enhancer, typically located between a Jκ gene and the

5   Cκ gene, or located downstream of the Cκ gene. For example,

the mouse heavy chain J-μ intronic enhancer (Banerji et al.

(1983) Cell 33: 729) can be isolated on a 0.9 kb XbaI fragment

of the plasmid pKVe2 (see, infra). The human heavy chain J-μ

intronic enhancer (Hayday et al. (1984) Nature 307: 334)

10 can be isolated as a 1.4 kb MluI/HindIII fragment (see,

infra). Addition of a transcriptionally active xenoenhancer

to a transgene, such as a combined xenoenhancer consisting

essentially of a human J-μ intronic enhancer linked in cis to

a mouse J-μ intronic enhancer, can confer high levels of

15 expression of the transgene, especially where said transgene

encodes a light chain, such as human κ. Similarly, a rat 3'

enhancer can be advantageously included in a minilocus

construct capable of encoding a human heavy chain.

20 Specific Preferred Embodiments

        A preferred embodiment of the invention is an animal

containing at least one, typically 2-10, and sometimes 25-50

or more copies of the transgene described in Example 12 (e.g.,

pHC1 or pHC2) bred with an animal containing a single copy of

25 a light chain transgene described in Examples 5, 6, 8, or 14,

and the offspring bred with the J$_H$ deleted animal described in

Example 10. Animals are bred to homozygosity for each of

these three traits. Such animals have the following genotype:

a single copy (per haploid set of chromosomes) of a human

30 heavy chain unrearranged mini-locus (described in Example 12),

a single copy (per haploid set of chromosomes) of a rearranged

human κ light chain construct (described in Example 14), and a

deletion at each endogenous mouse heavy chain locus that removes all of the functional J_H segments (described in Example 10). Such animals are bred with mice that are homozygous for the deletion of the J_H segments (Examples 10) to produce

5   offspring that are homozygous for the J_H deletion and hemizygous for the human heavy and light chain constructs. The resultant animals are injected with antigens and used for production of human monoclonal antibodies against these antigens.

10   B cells isolated from such an animal are monospecific with regard to the human heavy and light chains because they contain only a single copy of each gene. Furthermore, they will be monospecific with regards to human or mouse heavy chains because both endogenous mouse heavy

15   chain gene copies are nonfunctional by virtue of the deletion spanning the J_H region introduced as described in Example 9 and 12. Furthermore, a substantial fraction of the B cells will be monospecific with regards to the human or mouse light chains because expression of the single copy of the rearranged

20   human κ light chain gene will allelically and isotypically exclude the rearrangement of the endogenous mouse κ and λ chain genes in a significant fraction of B-cells.

The transgenic mouse of the preferred embodiment will exhibit immunoglobulin production with a significant

25   repertoire, ideally substantially similar to that of a native mouse. Thus, for example, in embodiments where the endogenous Ig genes have been inactivated, the total immunoglobulin levels will range from about 0.1 to 10 mg/ml of serum, preferably 0.5 to 5 mg/ml, ideally at least about 1.0 mg/ml.

30   When a transgene capable of effecting a switch to IgG from IgM has been introduced into the transgenic mouse, the adult mouse ratio of serum IgG to IgM is preferably about 10:1. Of

course, the IgG to IgM ratio will be much lower in the immature mouse. In general, greater than about 10%, preferably 40 to 80% of the spleen and lymph node B cells express exclusively human IgG protein.

The repertoire will ideally approximate that shown in a non-transgenic mouse, usually at least about 10% as high, preferably 25 to 50% or more. Generally, at least about a thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending primarily on the number of different V, J and D regions introduced into the mouse genome. These immunoglobulins will typically recognize about one-half or more of highly antigenic proteins, including, but not limited to: pigeon cytochrome C, chicken lysozyme, pokeweed mitogen, bovine serum albumin, keyhole limpit hemocyanin, influenza hemagglutinin, staphylococcus protein A, sperm whale myoglobin, influenza neuraminidase, and lambda repressor protein. Some of the immunoglobulins will exhibit an affinity for preselected antigens of at least about $10^7 M^{-1}$, preferably $10^8 M^{-1}$ to $10^9 M^{-1}$ or greater.

In some embodiments, it may be preferable to generate mice with predetermined repertoires to limit the selection of V genes represented in the antibody response to a predetermined antigen type. A heavy chain transgene having a predetermined repertoire may comprise, for example, human V_H genes which are preferentially used in antibody responses to the predetermined antigen type in humans. Alternatively, some V_H genes may be excluded from a defined repertoire for various reasons (e.g., have a low likelihood of encoding high affinity V regions for the predetermined antigen; have a low propensity to undergo somatic mutation and affinity sharpening; or are immunogenic to certain humans).

Thus, prior to rearrangement of a transgene
containing various heavy or light chain gene segments, such
gene segments may be readily identified, e.g. by hybridization
or DNA sequencing, as being from a species of organism other
5   than the transgenic animal.

The transgenic mice of the present invention can be
immunized with a predetermined antigen, such as a
transmembrane proteins, cell surface macromolecule, or other
suitable antigen (e.g., TNF, LPS, etc.) for which a human
10   antibody would be desirable. The mice will produce B cells
which undergo class-switching via intratransgene switch
recombination (cis-switching) and express immunoglobulins
reactive with the predetemined antigen. The immunoglobulins
can be human sequence antibodies, wherein the heavy and light
15   chain polypeptides are encoded by human transgene sequences,
which may include sequences derived by somatic mutation and V
region recombinatorial joints, as well as germline-encoded
sequences; these human sequence immunoglobulins can be
referred to as being substantially identical to a polypeptide
20   sequence encoded by a human V_L or V_H gene segment and a human
J_L or J_L segment, even though other non-germline sequences may
be present as a result of somatic mutation and differential V-
J and V-D-J recombination joints. With respect to such human
sequence antibodies, the variable regions of each chain are
25   typically at least 80 percent encoded by human germline V, J,
and, in the case of heavy chains, D, gene segments; frequently
at least 85 percent of the variable regions are encoded by
human germline sequences present on the transgene; often 90 or
95 percent or more of the variable region sequences are
30   encoded by human germline sequences present on the transgene.
However, since non-germline sequences are introduced by
somatic mutation and VJ and VDJ joining, the human sequence

antibodies will frequently have some variable region sequences (and less frequently constant region sequences) which are not encoded by human V, D, or J gene gegments as found in the human transgene(s) in the germline of the mice. Typically,

5   such non-germline sequences (or individual nucleotide positions) will cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

The human sequence antibodies which bind to the predetermined antigen can result from isotype switching, such

10 that human antibodies comprising a human sequence γ chain (such as γ1, γ2a, γ2B, or γ3) and a human sequence light chain (such as K) are produced. Such isotype-switched human sequence antibodies often contain one or more somatic mutation(s), typically in the variable region and often in or

15 within about 10 residues of a CDR) as a result of affinity maturation and selection of B cells by antigen, particualarly subsequent to secondary (or subsequent) antigen challenge. These high affinity human sequence antibodies may have binding affinities of at least 1×10^9 M^{-1}, typically at least

20 $5 \times 10^9 M^{-1}$, frequently more than 1×10^{10} M^{-1}, and sometimes $5 \times 10^{10}M^{-1}$ to 1×10^{-11} or greater. Such high affinity human sequence antibodies can be made with high binding affinities for human antigens, such as human CD4 and the like human macromolecules (e.g., such as a human transmembrane or cell

25 surface protein or other cell surface antigen).

The B cells from such mice can be used to generate hybridomas expressing monoclonal high affinity (greater than 2×10^9 M^{-1}) human sequence antibodies against a variety of antigens, including human proteins such as CD4 and the like.

30 These hybridomas can be used to generate a composition comprising an immunoglobulin having an affinity constant (K_a)

of at least 2 x 10⁹ M⁻¹ for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

Often, the human sequence heavy chain and human sequence light chain are separately encoded by a human heavy chain transgene and a human light chain transgene, respectively, which are integrated into a mouse cell genome. However, both chains may be encoded on a single transgene, or one or both chains may be encoded on multiple transgenes, such as a human heavy chain transgene (e.g., HC2) which derived a V gene segment from a YAC containing a V_H array which is not integrated ar the same locus as the human heavy chain transgene in the mouse germline.

In one embodiment, the composition has an immunoglobulin which comprises a human sequence light chain having a κ constant region and a human sequence heavy chain having a γ constant region.

The mice (and hybridomas derived therefrom) are a source for an immunoglobulin having an affinity constant (K_a)

of at least 1 x10^{10} M^{-1} for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

The invention provides a transgenic mouse comprising: a homozygous pair of functionally disrupted endogenous heavy chain alleles, a homozygous pair of functionally disrupted endogenous light chain alleles, at least one copy of a heterologous immunoglobulin light chain transgene, and at least one copy of a heterologous immunoglobulin heavy chain transgene, and wherein said animal makes an antibody response following immunization with a human antigen wherein the antibody response comprises an immunoglobulin having an affinity constant (K$_a$) of at least 2 x 10^9 M^{-1} for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light

chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a

5 heavy chain variable region having a polypeptide sequene which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide

10 sequence encoded by a human C_H gene segment.

Such a transgenic mouse can produce a human sequence immunoglobulin which binds to a human surface or transmembrane protein present on at least one somatic cell type of a human, wherein the immunoglobulin binds said human surface or

15 transmembrane protein with an affinity constant (K_a) of between 1.5×10^9 M^{-1} and 1.8×10^{10} M^{-1}. One example of such a human surface or transmemebrane protein is CD4, although others may be used as immunogens as desired.

The development of high affinity human sequence

20 antibodies against predetermined antigens is facilitated by a method for expanding the repertoire of human variable region gene segments in a transgenic mouse having a genome comprising an integrated human immunoglobulin transgene, said method comprising introducing into the genome a V gene transgene

25 comprising V region gene segments which are not present in said integrated human immunoglobulin transgene. Often, the V region transgene is a yeast artificial chromosome comprising a portion of a human V_H or V_L (V_k) gene segment array, as may naturally occur in a human genome or as may be spliced

30 together separately by recombinant methods, which may include out-of-order or omitted V gene segments. Often at least five or more functional V gene segments are contianed on the YAC.

In this variation, it is possible to make a transgenic mouse produced by the V repertoire expansion method, wherein the mouse expresses an immunoglobulin chain comprising a variable region sequence encoded by a V region gene segment present on

5 the V region transgene and a C region encoded on the human Ig transgene. By means of the V repertoire expansion method, transgenic mice having at least 5 distinct V genes can be generated; as can mice containing at least about 24 V genes or more. Of course, some V gene segments may be non-functional

10 (e.g., pseudogenes and the like); these segments may be retained or may be selectively deleted by recombinant methods avaialble to the skilled artisan, if desired.

Once the mouse germline has been engineered to contain a functional YAC having an expanded V segment

15 repertoire, substantially not present in the human Ig transgene containing the J and C gene segments, the trait can be propagated and bred into other genetic backgrounds, including backgrounds where the functional YAC having an expanded V segment repertoire is bred into a mouse germline

20 having a different human Ig transgene. Multiple functional YACs having an expanded V segment repertoire may be bred into a germline to work with a human Ig transgene (or multiple human Ig transgenes). Although referred to herein as YAC transgenes, such transgenes when integrated into the genome

25 may substantially lack yeast sequences, such as sequences required for autonomous replication in yeast; such sequences may optionally be removed by genetic engineering (e.g., restriction digestion and pulsed-field gel electrophoresis or other suitable method) after replication in yeast in no longer

30 necessary (i.e., prior to introduction into a mouse ES cell or mouse prozygote).

The invention also provides a method of propagating the trait of human sequence immunoglobulin expression, comprising breeding a transgenic mouse having the human Ig transgene(s), and optionally also having a functional YAC

5 having an expanded V segment repertoire. Both V_H and V_L gene segemnts may be present on the YAC. The transgenic mouse may be bred into any background desired by the practitioner, including backgrounds harboring other human transgenes, including human Ig transgenes and/or transgenes encoding other

10 human lymphocyte proteins.

The invention also provides a high affinity human sequence immunoglobulin produced by a transgenic mouse having an expanded V region repertoire YAC transgene.

Although the foregoing describes a preferred

15 embodiment of the transgenic animal of the invention, other embodiments are defined by the disclosure herein and more particularly by the transgenes described in the Examples. Four categories of transgenic animal may be defined:

I. Transgenic animals containing an unrearranged heavy

20 and rearranged light immunoglobulin transgene.

II. Transgenic animals containing an unrearranged heavy and unrearranged light immunoglobulin transgene

III. Transgenic animal containing rearranged heavy and an unrearranged light immunoglobulin transgene, and

25 IV. Transgenic animals containing rearranged heavy and rearranged light immunoglobulin transgenes.

Of these categories of transgenic animal, the preferred order of preference is as follows II > I > III > IV where the endogenous light chain genes (or at least the κ

30 gene) have been knocked out by homologous recombination (or other method) and I > II > III >IV where the endogenous light

chain genes have not been knocked out and must be dominated by allelic exclusion.

As is discussed supra, the invention provides human sequence monoclonal antibodies that are useful in treatment of human diseases. Therapeutic uses of monoclonal antibodies are discussed in, e.g., Larrick and Bourla, *Journal of Biological Response Modifiers*, 5:379-393, which is incorporated herein by reference. Uses of human monoclonal antibodies include treatment of autoimmune diseases, cancer, infectious diseases, transplant rejection, blood disorders such as coagulation disorders, and other diseases.

The antibodies of this invention may be administered to patients by any method known in the medical arts for delivery of proteins. Antibodies are particularly suited for parenteral administration (i.e, subcutaneous, intramuscular or intravenous administration). The pharmaceutical compositions of the present invention are suitable for administration using alternative drug delivery approaches as well (*see*, e.g., Langer, *Science*, 249:1527-1533 (1990)).

Pharmaceutical compositions for parenteral administration usually comprise a solution of a monoclonal antibody dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH-adjusting and buffering agents, tonicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium

lactate, etc. The concentration of antibody in these
formulations can vary widely, i.e., from less than about 0.5%,
usually at or at least about 0.1% to as much as 1.5% or 2.0%
by weight and will be selected primarily based on fluid

5 volumes, viscosities, etc., in accordance with the particular
mode of administration selected. Actual methods for preparing
parenterally administrable compositions will be known or
apparent to those skilled in the art and are described in more
detail in, for example, Remington's Pharmaceutical Sciences,

10 17th Ed., Mack Publishing Company, Easton, Pennsylvania
(1985), which is incorporated herein by reference.

The compositions containing the present antibodies
or a cocktail thereof can be administered for the prophylactic
and/or therapeutic treatments. In therapeutic application,

15 compositions are administered to a patient in an amount
sufficient to cure or at least partially arrest the infection
and its complications. An amount adequate to accomplish this
is defined as a "therapeutically effective dose." Amounts
effective for this use generally range from about .05 mg/kg

20 body weight to about 5 mg/kg body weight, preferably between
about .2 mg/kg body weight to about 1.5 mg/kg body weight.

In some instances it will be desirable to modify the
immunoglobulin molecules of the invention to change their
biological activity. For example, the immunoglobulins can be

25 directly or indirectly coupled to other chemotherapeutics
agent. A variety of chemotherapeutics can be coupled for
targeting. For example, anti-inflammatory agents which may be
coupled include immunomodulators, platelet activating factor
(PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase

30 inhibitors, and leukotriene antagonists. Some preferred
moieties include cyclosporin A, indomethacin, naproxen, FK-
506, mycophenolic acid, and the like. Similarly, anti-

oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury. Likewise, anticancer agents, such as daunomycin, doxorubicin, vinblastine, bleomycin, and the like can be targeted.

The monoclonal antibodies of the invention may also be used to target amphipaths (e.g., liposomes) to sites in a patient. In these preparations, the drug to be delivered is incorporated as part of a liposome in which a human monoclonal antibody is embedded.

The human-sequence monoclonal antibodies of the invention are useful, in part, because they bind specifically to the predetermined antigen against which they are directed. When the predetermined antigen is a human antigen (i.e., a human protein or fragment thereof), it will sometimes be advantageous if the human immunoglobulin of the invention also binds to the *cognate* antigen found in non-human animals, especially animals that are used frequently for drug testing (e.g., preclinical testing of biological activity, pharmacokinetics and safety). These animals include mice, rabbits, rats, dogs, pigs, and, especially, non-human primates such as chimpanzees, apes and monkeys (e.g., Rhesus monkeys and cynomolgus monkeys). The ability to recognize antigens in experimental animals is particularly useful for determining the effect of specific binding on biodistribution of the immunoglobulins. A cognate antigen is an antigen that (i) has a structure (e.g., amino acid sequence) that is substantially similar to the human antigen (i.e., the amino acid sequence of an animal cognate protein will typically be at least about 50% identical to the human protein, usually at least about 70% identical and often at least about 80% identical or more); (ii) has substantially the same function as the human antigen; and, (iii) often is found in the same cellular compartment as

the human antigen. Human and animal cognate antigens typically
(but not always) have the same names. Examples of cognate
antigens include human tubulin and mouse tubulin, human CD4
and Rhesus CD4, and human IgG and Rat IgG.

5   An other aspect, the invention provides antigen-
binding human mABs comprising at least one polypeptide encoded
by an artificial gene. An artificial gene comprises a
polypeptide-encoding nucleic acid segment that is synthesized
in vitro by chemical or enzymatic methods that do not require
10 a cell-derived template nucleic acid strand (e.g., a nucleic
acid template obtained from a bacterial cell or an immune or
hybridoma cell) and the progeny (through replication) of the
artificial gene, i.e., a wholly synthetic nucleic acid.

          Although it is routine in genetic engineering to use
15 short synthetic nucleic acids as primers, linkers and the
like, it is also possible by chemical and/or enzymatic means
to produce wholly synthetic protein-coding nucleic acids that
are 30, 50, or more bases in length. The artificial genes of
the invention may include both synthetic nucleic acid regions
20 and cell-derived nucleic acid regions. The synthetic nucleic
acid region of the artificial gene will generally be at least
about 50 bases in length, often at least about 100 bases,
typically at least about 200 bases, more often at least about
250 bases and usually over 300 bases or 400 bases in length.
25 Typically the synthetic nucleic acid regions will encode
variable gene segments or a portion thereof, e.g., CDR
regions, and the constant regions will be encoded by cell-
derived nucleic acids.   Immunoglobulin polypeptides (i.e.,
immunoglobulin heavy chains and immunoglobulin light chains)
30 can be conveniently expressed using artificial genes that
encode the polypeptides. Usually the artificial genes are
operably linked to transcription promoter sequences, e.g.,

promoter sequences derived from immunoglobulin genes or from viruses (e.g., SV40, CMV, HIV, RSV) or hybrid promoters. The artificial gene may be linked to other sequences as well, e.g. polyadenylation sequences and introns. One method for

5   expressing an immunoglobulin polypeptide involves insertion of a synthetic nucleic acid encoding one region of an immunoglobulin polypeptide (e.g., a variable region or portion thereof) into a vector that encodes the remaining segments or parts of the immunoglobulin chain (e.g., a μ, γ, $\gamma2$, $\gamma3$, $\gamma4$,

10 δ, ϵ, α_1 or α_2 constant region) and, optionally, promoter (e.g., a CMV (cytomegalovirus) promoter), polyadenylation or other sequences. Such vectors are constructed so that upon introduction into a cell, the cellular transcription and translation of the vector sequences results in an immunoglobin

15 polypeptide.

Functional human sequence immunoglobulin heavy and light chain genes and polypeptides can be constructed using artificial genes, and used to produce immunoglobulins with a desried specificity such as specific binding to a

20 predetermined antigen. This is accomplished by constructing an artificial gene that encodes an immunoglobulin polypeptide substantially similar to a polypeptide expressed by a cell from, or a hybridoma derived from, a transgenic animal immunized with the predetermined antigen. Thus, the invention

25 provides artificial genes encoding immunoglobulin polypeptides and methods for producing a human-sequence immunoglobulin using an artificial gene(s).

According to this method, a transgenic animal (e.g., a transgenic mouse with a homozygous pair of functionally

30 disrupted endogenous heavy chain alleles, a homozygous pair of functionally disrupted endogenous light chain alleles, at least one copy of a human immunoglobulin light chain

transgene, and at least one copy of a human immunoglobulin

heavy chain transgene) is immunized with predetermined

antigen, e.g., a human protein. Nucleic acid, preferably

mRNA, is then collected or isolated from a cell or population

5   of cells in which immunoglobulin gene rearrangement has taken

place, and the sequence(s) of nucleic acids encoding the heavy

and/or light chains (especially the V segments) of

immunoglobulins, or a portion thereof, is determined. This

sequence information is used as a basis for the sequence of

10   the artificial gene.

Sequence determination will generally require

isolation of at least a portion of the gene or cDNA of

interest, e.g., a portion of a rearranged human transgene or

corresponding cDNA encoding an immunoglobulin polypeptide.

15   Usually this requires cloning the DNA or, preferably, mRNA

(i.e., cDNA) encoding the human immunoglobulin polypeptide.

Cloning is carried out using standard techniques (see, e.g.,

Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Guide*,

Vols 1-3, Cold Spring Harbor Press, which is incorporated

20   herein by reference). For example, a cDNA library may be

constructed by reverse transcription of polyA+ mRNA,

preferably membrane-associated mRNA, and the library screened

using probes specific for human immunoglobulin polypeptide

gene sequences. In a preferred embodiment, however, the

25   polymerase chain reaction (PCR) is used to amplify cDNAs (or

portions of full-lenght cDNAs) encoding an immunoglobulin gene

segment of interest (e.g., a light chain variable segment).

Because the sequences of the human immunoglobulin polypeptide

genes are readily available to those of skill, probes or PCR

30   primers that will specifically hybridize to or amplify a human

immunoglobulin gene or segment thereof can be easily designed.

See, e.g., Taylor *et al., Nuc. Acids. Res.,* 20:6287 (1992)

which is incorporated by reference. Moreover, the sequences of the human transgene of the transgenic mouse will often be known to the practicioner, and primer sequences can be chosen that hybridize to appropriate regions of the transgene. The

5 amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used not critical, so long as it is possible to determine the sequence of some portion of the

10 immunoglobulin polypeptide of interest. As used herein, a nucleic acid that is cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the seqence of the nucleic acid of interest can be determined, is considered isolated.

15   One source for RNA used for cloning and sequencing is a hybridoma produced by obtaining a B cell from the transgenic mouse and fusing the B cell to an immortal cell. An advantage of using hybridomas is that they can be easily screened, and a hybridoma that produces a human monoclonal

20 antibody of interest selected. Alternatively, RNA can be isolated from B cells (or whole spleen) of the immunized animal. When sources other than hybridomas are used, it may be desirable to screen for sequences encoding immunoglobulins or immunoglobulin polypeptides with specific binding

25 characteristics. One method for such screening is the use of phage display technology. Phage display is described in e.g., Dower et al., WO 91/17271, McCafferty et al., WO 92/01047, and Caton and Koprowski, Proc. Natl. Acad. Sci. USA, 87:6450-6454 (1990), each of which is incorporated herein by reference. In

30 one embodiment using phage display technology, cDNA from an immunized transgenic mouse (e.g., total spleen cDNA) is isolated, the polymerase chain reaction is used to amplify a

cDNA sequences that encode a portion of an immunoglobulin polypeptide, e.g., CDR regions, and the amplified sequences are inserted into a phage vector. cDNAs encoding peptides of interest, e.g., variable region peptides with desired binding

5   characteristics, are identified by standard techniques such as panning.

The sequence of the amplified or cloned nucleic acid is then determined. Typically the sequence encoding an entire variable region of the immunoglobulin polypeptide is

10 determined, however, it will sometimes by adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Typically the portion sequenced will be at least 30 bases in length, more often based coding for at least about one-third or aty least about one-half of the length of

15 the variable region will be sequenced.

Sequencing can be carried on clones isolated from a cDNA library, or, when PCR is used, after subcloning the amplified sequence or by direct PCR sequencing of the amplified segment. Sequencing is carried out using standard

20 techniques (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with

25 published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light

30 chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the

National Center for Biotechnology Information, National
Library of Medicine, National Institutes of Health, Bethesda,
Md.

5   In an alternative embodiment, the amino acid
sequence of an immunoglobulin of interest may be determined by
direct protein sequencing.

An artificial gene can be constructed that has a
sequence identical to or substantially similar to, at least a
portion of the immunoglobulin-expressing gene (i.e.,
10   rearranged transgene). Similarly, the artificial gene can
encode an polypeptide that is identical or has substantial
similarity to a polypeptide encoded by the sequenced portion
of the rearranged transgene. The degeneracy of the genetic
code allows the same polypeptide to be encoded by multiple
15   nucleic acid sequences. It is sometimes desirable to change
the nucleic acid sequence, for example to introduce
restriction sites, change codon usage to reflect a particular
expression system, or to remove a glycosylation site. In
addition, changes in the hybridoma sequences may be introduced
20   to change the characteristics (e.g., binding characteristics)
of the immunoglobulin. For example, changes may be
introduced, especially in the CDR regions of the heavy and
light chain variable regions, to increase the affinity of the
immunoglobulin for the predetermined antigen.

25   Methods for constructing an synthetic nucleic acids
are well known. An entirely chemical synthesis is possible
but in general, a mixed chemical-enzymatic synthesis is
carried out in which chemically synthesized oligonucleotides
are used in ligation reactions and/or in the polymerase chain
30   reaction to create longer polynucleotides. In a most
preferred embodiment, the polymerase chain reaction is carried
out using overlapping primers chosen so that the result of the

amplification is a DNA with the sequence desired for the artificial gene. The oligonucleotides of the present invention may be synthesized in solid phase or in solution. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of oligonucleotides by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage et al., *Tetrahedron Lett.*, 22:1859-1862; Matteucci et al., *J. Amer. Chem. Soc.*, 103:3185-3191 (1981); Caruthers et al., *Genetic Engineering*, 4:1-17 (1982); Jones, chapter 2, Atkinson et al., chapter 3, and Sproat et al., chapter 4, in Gait, ed. *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Washington, D.C. (1984); Froehler et al., *Tetrahedron Lett.*, 27:469-472 (1986); Froehler et al., *Nucleic Acids Res.*, 14:5399-5407 (1986); Sinha et al., *Tetrahedron Lett.*, 24:5843-5846 (1983); and Sinha et al., *Nucleic Acids Res.*, 12:4539-4557 (1984) which are incorporated herein by reference.

The artificial gene can introduced into a cell and expressed to produce an immunoglobulin polypeptide. The choice of cell type for expression will depend on many factors (e.g., the level of protein glycosylation desired), but cells capable of secreting human immunoglobulins will be preferred. Especially preferred cells include CHO cells and myeloma-derived cells such as the SP20 and NS0 cell lines. Standard cell culture are well known and are also described in Newman, et al., *Biotechnology*, 10:1455-1460 (1992); Bebbington, et al., *Biotechnology*, 10:169-175 (1992); Cockett, et al., *Biotechnology*, 8:662-667 (1990); Carter, et al., *Biotechnology*, 10:163-167 (1992), each of which is incorporated herein by reference. Methods for introduction of

nucleic acids, e.g., an artificial gene, are well known and
include transfection (e.g., by electroporation or liposome-
mediated) and transformation. Systems for expression of
introduced genes are described generally in Sambrook et al.,
5   *supra*.

It is often desirable to express two immunoglobulin
polypeptides (i.e., a heavy chain and a light chain) in the
same cell so that an immunoglobulin (e.g., an IgG molecule) is
produced *in vivo*. Accordingly it will sometimes be desirable
10 to introduce two artificial genes (i.e., one encoding a heavy
chain and one encoding a light chain) into a cell. (The two
artificial genes can be introduced on a single vector).
Alternatively, one artificial gene encoding one immunoglobulin
polypeptide can be introduced into a cell that has been
15 genetically engineered to express the other immunoglobulin
polypeptide.

It will be apparent that as the cells into which the
artificial gene is transfected propagate, the wholly synthetic
nucleic acid portion of the artificial gene, will act as a
20 template for replication and transcription. Nonetheless, the
progeny genes will have originated from a synthetic nucleic
acid (i.e., a polypeptide-encoding nucleic acid molecule that
is synthesized *in vitro* by chemical or enzymatic methods that
do not require a cell-derived template nucleic acid strand)
25 and as used herein, are also considered artificial genes.
Thus, the relationship of the synthetic portion of the
artificial gene to the expressed transgene of the hybridoma is
one in which there is an *informational* link (i.e., sequence
information) but no direct *physical* link.

30   The invention also provides anti-CD4 monoclonal
antibodies useful in therapeutic and diagnostic applications,
especially the treatment of human disease. CD4 is a cell

surface protein that is expressed primarily on thymocytes and T cells, and which is involved in T-cell function and MHC Class II recognition of antigen. Antibodies directed against CD4 act to reduce the activity of CD4 cells and thus reduce

5 undesirable autoimmune reactions, inflammatory responses and rejection of transplanted organs.

The ability of a human anti-CD4 mAb to inhibit a T-helper cell dependent immune response in primates can be demonstrated by immunizing the primate with a soluble foreign

10 antigen (e.g., tetanus toxoid (TT)) and measuring the ability of the primate to mount a delayed-type hypersensitivity reaction (DTH) to the antigen (e.g., following injection of the human mAb). The DTH is mediated by CD4$^+$ (T-helper) cells (E. Benjamin and S. Lescowitz, Immunology: A Short Course,

15 Second Edition, (1991) Wiley-Liss, Inc., New York, pp. 277-292). Antigen-specific T-helper cells recognize the processed antigen presented by MHC Class II molecules on antigen-presenting cells and become activated. The activated T-helper cells secrete a variety of lymphokines (IL2, INFγ, TNFβ, MCF)

20 and thus attract and activate macrophages and T-cytotoxic cells at the injection site. Although most of the effector functions occurring as part of the DTH are performed by macrophages and T-cytotoxic cells, it is the T-helper cells which initiate the response. Therefore, if the T-helper cells

25 can be inhibited, there will be no DTH. Administration of anti-CD4 mABs has been shown to prevent (Wofsy, et al., J. Exp. Med., 161:378-391 (1985)) or reverse (Wofsy, et al., J. Immunol., 138:3247-3253 (1987), Waldor, et al., Science, 227:415-417 (1985)) autoimmune disease in animal models.

30 Administration of murine or chimeric anti-CD4 mAbs to patients with rheumatoid arthritis has shown evidence of clinical benefit (Knox, et al., Blood, 77:20-30 (1991); Goldbery, et

al., *J. Autoimmunity*, 4:617-630; Herzog, et al., *Lancet*,
ii:1461-1462 ; Horneff, et al., *Arthritis Rheum.*, 34:129-140;
Reiter, et al., *Arthritis Rheum.*, 34:525-536; Wending, et al.,
J. Rheum., 18:325-327; Van der Lubbe, et al., *Arthritis
Rheum.*, 38:1097-1106; Van der Lubbe, et al., *Arthritis Rheum.*,
36:1375-1379; Moreland, et al., *Arthritis Rheum.*, 36:307-318,
and Choy, et al., *Arthritis and Rheumatism*, 39(1):52-56
(1996); all of which is incorporated herein by reference). In
addition, as noted above, a chimeric anti-CD4 mAB has shown
some clinical efficacy in patients with mycosis fungoides
(Knox et al. (1991) Blood 77:20; which is incorporated herein
by reference). Anti-CD4 antibodies are also discussed in
Newman, et al., *Biotechnology*, 10:1455-1460 (1992), which is
incorporated herein by reference.

The invention also provides anti-interleukin-8
monoclonal antibodies useful in therapeutic and diagnostic
applications, especially the treatment of human diseases.
Interleukin-8 (IL8), a very potent and mostly specific
chemoattractant for neutrophils, is thought to play an
important role in the inflammatory response. IL8 also induces
angiogenesis, mediates transendothelial neutrophil migration
and contributes to other inflammatory responses. The
properties of IL8 and related cytokines are discussed in
Baggiolini *et al.*, 1994, *Adv. Immunol.* 55:97-179, which is
incorporated herein by reference.

IL8 has been shown to bind to, and activate,
neutrophils and to induce neutrophil chemotaxis through an
endothelial cell layer *in vitro*. The role of IL8 in inducing
neutrophil transmigration from the vasculature to a site of
inflammation has been demonstrated *in vivo* as well. Moreover,
inhibition of the IL8 in those circumstances has prevented
tissue damage resulting from neutrophil recruitment. Anti-

rabbit IL8 antibodies can prevent lung reperfusion injuries resulting from ischemia (Sekido et al., 1993, Nature 365:654-7). Anti-rabbit IL8 antibodies can also prevent lung damage resulting from endotoxin-induced pleurisy in rabbits (Broaddus

5   et al., 1994, J. Immunol. 152:2960-7). In vivo primate models are also suitable for determing the effects of anti-human IL8 mAbs on migration of neutrophils from the vasculature to the inflammatory site. It has been shown that intradermal injection of rhesus monkeys with endotoxin upregulates IL8

10 expression at the injection site and ultimately results in neutrophil localization (Silber et al., 1994, Lab. Invest. 70:163-75).

        Anti-IL8 antibodies have also been shown to reduce tissue damage and prolong survival in animal models of acute

15 inflammation including adult respiratory distress syndrome (ARDS) and acid induced lung injury (Sekido et al., 1993, Nature 365:654-7; Mulligan et al., 1993, J. Immunol. 150:5585-95; Broaddus et al., 1994, J. Immunol. 152:2960-7, all of which are incorporated herein by reference).

20 Consistent with the role of IL-8 in inflammation, human anti-IL-8 monoclonal antibodies can be used for treatment of a variety of conditions caused or aggravated by an inflammatory response, including reperfusion injuries (especially to the lung and heart), vasculitis, septic shock, autoimmune diseases

25 (including glomerulonephritis, inflammatory bowel disease, rheumatoid arthritis and psoriasis), allergic reactions (e.g., asthma) and cystic fibrosis.

        Two distinct IL8 receptors, IL8RA and IL8RB, have been identified (Holmes et al., 1991, Science 253:1278-80;

30 Murphy et al., 1991, Science 253:1280-3). Both receptors bind IL8, but only IL8RB binds other CXC chemokines. Both

receptors are found in approximately equal numbers on neutrophils and some lymphocytes.

EXPERIMENTAL EXAMPLES

5 <u>METHODS AND MATERIALS</u>

Transgenic mice are derived according to Hogan, et al., "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory, which is incorporated herein by reference.

10   Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, ed., IRL Press, Washington, D.C., 1987; Zjilstra et al., <u>Nature</u> <u>342</u>:435-438 (1989); and Schwartzberg et al., <u>Science</u> <u>246</u>:799-803 (1989),

15 each of which is incorporated herein by reference).

DNA cloning procedures are carried out according to J. Sambrook, et al. in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by

20 reference.

Oligonucleotides are synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Hybridoma cells and antibodies are manipulated

25 according to "Antibodies: A Laboratory Manual", Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference.

EXAMPLE 1

30   <u>Genomic Heavy Chain Human Ig Transgene</u>

This Example describes the cloning and microinjection of a human genomic heavy chain immunoglobulin transgene which is microinjected into a murine zygote.

Nuclei are isolated from fresh human placental

5   tissue as described by Marzluff et al., "Transcription and Translation: A Practical Approach", B.D. Hammes and S.J. Higgins, eds., pp. 89-129, IRL Press, Oxford (1985)). The isolated nuclei (or PBS washed human spermatocytes) are embedded in a low melting point agarose matrix and lysed with

10 EDTA and proteinase κ to expose high molecular weight DNA, which is then digested in the agarose with the restriction enzyme NotI as described by M. Finney in Current Protocols in Molecular Biology (F. Ausubel, et al., eds. John Wiley & Sons, Supp. 4, 1988, Section 2.5.1).

15   The NotI digested DNA is then fractionated by pulsed field gel electrophoresis as described by Anand et al., Nucl. Acids Res. 17:3425-3433 (1989). Fractions enriched for the NotI fragment are assayed by Southern hybridization to detect one or more of the sequences encoded by this fragment.

20 Such sequences include the heavy chain D segments, J segments, μ and γ1 constant regions together with representatives of all 6 VH families (although this fragment is identified as 670 kb fragment from HeLa cells by Berman et al. (1988), supra., we have found it to be as 830 kb fragment from human placental an

25 sperm DNA). Those fractions containing this NotI fragment (see Fig. 4) are pooled and cloned into the NotI site of the vector pYACNN in Yeast cells. Plasmid pYACNN is prepared by digestion of pYAC-4 Neo (Cook et al., Nucleic Acids Res. 16: 11817 (1988)) with EcoRI and ligation in the presence of the

30 oligonucleotide 5' - AAT TGC GGC CGC - 3'.

YAC clones containing the heavy chain NotI fragment are isolated as described by Brownstein et al., Science

244:1348-1351 (1989), and Green et al., Proc. Natl. Acad. Sci.
USA 87:1213-1217 (1990), which are incorporated herein by
reference. The cloned NotI insert is isolated from high
molecular weight yeast DNA by pulse field gel electrophoresis
as described by M. Finney, op cit. The DNA is condensed by
the addition of 1 mM spermine and microinjected directly into
the nucleus of single cell embryos previously described.

EXAMPLE 2

Genomic κ Light Chain Human Ig Transgene
Formed by In Vivo Homologous Recombination

A map of the human κ light chain has been described
in Lorenz et al., Nucl. Acids Res. 15:9667-9677 (1987), which
is incorporated herein by reference.

A 450 kb XhoI to NotI fragment that includes all of
C_κ, the 3' enhancer, all J segments, and at least five
different V segments is isolated and microinjected into the
nucleus of single cell embryos as described in Example 1.

EXAMPLE 3

Genomic κ Light Chain Human Ig Transgene
Formed by In Vivo Homologous Recombination

A 750 kb MluI to NotI fragment that includes all of
the above plus at least 20 more V segments is isolated as
described in Example 1 and digested with BssHII to produce a
fragment of about 400 kb.

The 450 kb XhoI to NotI fragment plus the
approximately 400 kb MluI to BssHII fragment have sequence
overlap defined by the BssHII and XhoI restriction sites.
Homologous recombination of these two fragments upon
microinjection of a mouse zygote results in a transgene

containing at least an additional 15-20 V segments over that
found in the 450 kb XhoI/NotI fragment (Example 2).

EXAMPLE 4

Construction of Heavy Chain Mini-Locus

A. Construction of pGP1 and pGP2

pBR322 is digested with EcoRI and StyI and ligated
with the following oligonucleotides to generate pGP1 which
contains a 147 base pair insert containing the restriction
sites shown in Fig. 8. The general overlapping of these
oligos is also shown in Fig. 9.

The oligonucleotides are:

oligo-1 5' - CTT GAG CCC GCC TAA TGA GCG GGC TTT
              TTT TTG CAT ACT GCG GCC - 3'

oligo-2 5' - GCA ATG GCC TGG ATC CAT GGC GCG CTA
              GCA TCG ATA TCT AGA GCT CGA GCA -3'

oligo-3 5' - TGC AGA TCT GAA TTC CCG GGT ACC AAG
              CTT ACG CGT ACT AGT GCG GCC GCT -3'

oligo-4 5' - AAT TAG CGG CCG CAC TAG TAC GCG TAA
              GCT TGG TAC CCG GGA ATT - 3'

oligo-5 5' - CAG ATC TGC ATG CTC GAG CTC TAG ATA
              TCG ATG CTA GCG CGC CAT GGA TCC - 3'

oligo-6 5' - AGG CCA TTG CGG CCG CAG TAT GCA AAA
              AAA AGC CCG CTC ATT AGG CGG GCT - 3'

This plasmid contains a large polylinker flanked by
rare cutting NotI sites for building large inserts that can be
isolated from vector sequences for microinjection. The
plasmid is based on pBR322 which is relatively low copy
compared to the pUC based plasmids (pGP1 retains the pBR322
copy number control region near the origin of replication).
Low copy number reduces the potential toxicity of insert

sequences. In addition, pGP1 contains a strong transcription
terminator sequence derived from trpA (Christie et al., Proc.
Natl. Acad. Sci. USA 78:4180 (1981)) inserted between the
ampicillin resistance gene and the polylinker. This further
5 reduces the toxicity associated with certain inserts by
preventing readthrough transcription coming from the
ampicillin promoters.

        Plasmid pGP2 is derived from pGP1 to introduce an
additional restriction site (SfiI) in the polylinker. pGP1 is
10 digested with MluI and SpeI to cut the recognition sequences
in the polylinker portion of the plasmid.

        The following adapter oligonucleotides are ligated
to the thus digested pGP1 to form pGP2.

15   5' CGC GTG GCC GCA ATG GCC A 3'

                5' CTA GTG GCC ATT GCG GCC A 3'

        pGP2 is identical to pGP1 except that it contains an
additional Sfi I site located between the MluI and SpeI sites.
20 This allows inserts to be completely excised with SfiI as well
as with NotI.

B.   Construction of pRE3 (rat enhancer 3')

        An enhancer sequence located downstream of the rat
25 constant region is included in the heavy chain constructs.

        The heavy chain region 3' enhancer described by
Petterson et al., Nature 344:165-168 (1990), which is
incorporated herein by reference) is isolated and cloned. The
rat IGH 3' enhancer sequence is PCR amplified by using the
30 following oligonucleotides:

5' CAG GAT CCA GAT ATC AGT ACC TGA AAC AGG GCT TGC 3'

5' GAG CAT GCA CAG GAC CTG GAG CAC ACA CAG CCT TCC 3'

5   The thus formed double stranded DNA encoding the 3'
enhancer is cut with BamHI and SphI and clone into BamHI/SphI
cut pGP2 to yield pRE3 (rat enhancer 3').

C.   Cloning of Human J-μ Region
10   A substantial portion of this region is cloned by
combining two or more fragments isolated from phage lambda
inserts. See Fig. 9.
           A 6.3 kb BamHI/HindIII fragment that includes all
human J segments (Matsuda et al., EMBO J., 7:1047-1051 (1988);
15 Ravetech et al.m Cell, 27:583-591 (1981), which are
incorporated herein by reference) is isolated from human
genomic DNA library using the oligonucleotide GGA CTG TGT CCC
TGT GTG ATG CTT TTG ATG TCT GGG GCC AAG.
           An adjacent 10 kb HindIII/BamII fragment that
20 contains enhancer, switch and constant region coding exons
(Yasui et al., Eur. J. Immunol. 19:1399-1403 (1989)) is
similarly isolated using the oligonucleotide:
CAC CAA GTT GAC CTG CCT GGT CAC AGA CCT GAC CAC CTA TGA
           An adjacent 3' 1.5 kb BamHI fragment is similarly
25 isolated using clone pMUM insert as probe (pMUM is 4 kb
EcoRI/HindIII fragment isolated from human genomic DNA library
with oligonucleotide:
           CCT GTG GAC CAC CGC CTC CAC CTT CAT
           CGT CCT CTT CCT CCT
30 mu membrane exon 1) and cloned into pUC19.
           pGP1 is digested with BamHI and BglII followed by
treatment with calf intestinal alkaline phosphatase.

Fragments (a) and (b) from Fig. 9 are cloned in the digested pGP1. A clone is then isolated which is oriented such that 5' BamHI site is destroyed by BamHI/Bgl fusion. It is identified as pMU (see Fig. 10). pMU is digested with

5   BamHI and fragment (c) from Fig. 9 is inserted. The orientation is checked with HindIII digest. The resultant plasmid pHIG1 (Fig. 10) contains an 18 kb insert encoding J and Cμ segments.

10   D.   Cloning of Cμ Region

pGP1 is digested with BamHI and HindIII is followed by treatment with calf intestinal alkaline phosphatase (Fig. 14). The so treated fragment (b) of Fig. 14 and fragment (c) of Fig. 14 are cloned into the BamHI/HindIII cut pGP1. Proper

15   orientation of fragment (c) is checked by HindIII digestion to form pCON1 containing a 12 kb insert encoding the Cμ region.

Whereas pHIG1 contains J segments, switch and μ sequences in its 18 kb insert with an SfiI 3' site and a SpeI 5' site in a polylinker flanked by NotI sites, will be used

20   for rearranged VDJ segments. pCON1 is identical except that it lacks the J region and contains only a 12 kb insert. The use of pCON1 in the construction of fragment containing rearranged VDJ segments will be described hereinafter.

25   E.   Cloning of γ-1 Constant Region (pREG2)

The cloning of the human γ-1 region is depicted in Fig. 16.

Yamamura et al., Proc. Natl. Acad. Sci. USA 83:2152-2156 (1986) reported the expression of membrane bound

30   human γ-1 from a transgene construct that had been partially deleted on integration. Their results indicate that the 3' BamHI site delineates a sequence that includes the

transmembrane rearranged and switched copy of the gamma gene
with a V-C intron of less than 5kb. Therefore, in the
unrearranged, unswitched gene, the entire switch region is
included in a sequence beginning less than 5 kb from the 5'

5   end of the first γ-1 constant exon. Therefore it is included
in the 5' 5.3 kb HindIII fragment (Ellison et al., Nucleic
Acids Res. 10:4071-4079 (1982), which is incorporated herein
by reference). Takahashi et al., Cell 29: 671-679 (1982),
which is incorporated herein by reference, also reports that

10   this fragment contains the switch sequence, and this fragment
together with the 7.7 kb HindIII to BamHI fragment must
include all of the sequences we need for the transgene
construct. An intronic sequence is a nucleotide sequence of
at least 15 contiguous nucleotides that occurs in an intron of

15   a specified gene.

Phage clones containing the γ-1 region are
identified and isolated using the following oligonucleotide
which is specific for the third exon of γ-I (CH3).

20   5' TGA GCC ACG AAG ACC CTG AGG

                    TCA AGT TCA ACT GGT ACG TGG 3'

A 7.7 kb HindIII to BglII fragment (fragment (a) in
Fig. 11) is cloned into HindIII/BglII cut pRE3 to form pREG1.

25   The upstream 5.3 kb HindIII fragment (fragment (b) in Fig. 11)
is cloned into HindIII digested pREG1 to form pREG2. Correct
orientation is confirmed by BamHI/SpeI digestion.

F.   Combining Cγ and Cμ

30   The previously described plasmid pHIG1 contains
human J segments and the Cμ constant region exons. To provide
a transgene containing the Cμ constant region gene segments,

pHIG1 was digested with SfiI (Fig. 10). The plasmid pREG2 was also digested with SfiI to produce a 13.5 kb insert containing human Cγ exons and the rat 3' enhancer sequence. These sequences were combined to produce the plasmid pHIG3' (Fig.

5 12) containing the human J segments, the human Cμ constant region, the human Cγ1 constant region and the rat 3' enhancer contained on a 31.5 kb insert.

A second plasmid encoding human Cμ and human Cγ1 without J segments is constructed by digesting pCON1 with SfiI

10 and combining that with the SfiI fragment containing the human Cγ region and the rat 3' enhancer by digesting pREG2 with SfiI. The resultant plasmid, pCON (Fig. 12) contains a 26 kb NotI/SpeI insert containing human Cμ, human γ1 and the rat 3' enhancer sequence.

15

G. Cloning of D Segment

The strategy for cloning the human D segments is depicted in Fig. 13. Phage clones from the human genomic library containing D segments are identified and isolated

20 using probes specific for diversity region sequences (Ichihara et al., EMBO J. 7:4141-4150 (1988)). The following oligonucleotides are used:

DXP1:   5' - TGG TAT TAC TAT GGT TCG GGG AGT TAT TAT
25   AAC CAC AGT GTC - 3'

DXP4:   5' - GCC TGA AAT GGA GCC TCA GGG CAC AGT GGG
                  CAC GGA CAC TGT - 3'

30 DN4:   5' - GCA GGG AGG ACA TGT TTA GGA TCT GAG GCC
                  GCA CCT GAC ACC - 3'

A 5.2 kb XhoI fragment (fragment (b) in Fig. 13) containing DLR1, DXP1, DXP'1, and DA1 is isolated from a phage clone identified with oligo DXP1.

A 3.2 kb XbaI fragment (fragment (c) in Fig. 13) containing DXP4, DA4 and DK4 is isolated from a phage clone identified with oligo DXP4.

Fragments (b), (c) and (d) from Fig. 13 are combined and cloned into the XbaI/XhoI site of pGP1 to form pHIG2 which contains a 10.6 kb insert.

This cloning is performed sequentially. First, the 5.2 kb fragment (b) in Fig. 13 and the 2.2 kb fragment (d) of Fig. 13 are treated with calf intestinal alkaline phosphatase and cloned into pGP1 digested with XhoI and XbaI. The resultant clones are screened with the 5.2 and 2.2 kb insert. Half of those clones testing positive with the 5.2 and 2.2 kb inserts have the 5.2 kb insert in the proper orientation as determined by BamHI digestion. The 3.2 kb XbaI fragment from Fig. 13 is then cloned into this intermediate plasmid containing fragments (b) and (d) to form pHIG2. This plasmid contains diversity segments cloned into the polylinker with a unique 5' SfiI site and unique 3' SpeI site. The entire polylinker is flanked by NotI sites.

H.   Construction of Heavy Chain Minilocus

The following describes the construction of a human heavy chain mini-locus which contain one or more V segments.

An unrearranged V segment corresponding to that identified as the V segment contained in the hybridoma of Newkirk et al., J. Clin. Invest. 81:1511-1518 (1988), which is incorporated herein by reference, is isolated using the following oligonucleotide:

5' - GAT CCT GGT TTA GTT AAA GAG GAT TTT

ATT CAC CCC TGT GTC - 3'

A restriction map of the unrearranged V segment is
5   determined to identify unique restriction sites which provide
upon digestion a DNA fragment having a length approximately 2
kb containing the unrearranged V segment together with 5' and
3' flanking sequences. The 5' prime sequences will include
promoter and other regulatory sequences whereas the 3'
10 flanking sequence provides recombination sequences necessary
for V-DJ joining. This approximately 3.0 kb V segment insert
is cloned into the polylinker of pGB2 to form pVH1.

    pVH1 is digested with SfiI and the resultant
fragment is cloned into the SfiI site of pHIG2 to form a
15 pHIG5'. Since pHIG2 contains D segments only, the resultant
pHIG5' plasmid contains a single V segment together with D
segments. The size of the insert contained in pHIG5 is 10.6
kb plus the size of the V segment insert.

    The insert from pHIG5 is excised by digestion with
20 NotI and SpeI and isolated. pHIG3' which contains J, Cμ and
cγ1 segments is digested with SpeI and NotI and the 3' kb
fragment containing such sequences and the rat 3' enhancer
sequence is isolated. These two fragments are combined and
ligated into NotI digested pGP1 to produce pHIG which contains
25 insert encoding a V segment, nine D segments, six functional J
segments, Cμ, Cγ and the rat 3' enhancer. The size of this
insert is approximately 43 kb plus the size of the V segment
insert.

30 I.   Construction of Heavy Chain Minilocus
         by Homologous Recombination

As indicated in the previous section, the insert of
pHIG is approximately 43 to 45 kb when a single V segment is
employed. This insert size is at or near the limit of that
which may be readily cloned into plasmid vectors. In order to
5   provide for the use of a greater number of V segments, the
following describes in vivo homologous recombination of
overlapping DNA fragments which upon homologous recombination
within a zygote or ES cell form a transgene containing the rat
3' enhancer sequence, the human Cμ, the human Cγ1, human J
10 segments, human D segments and a multiplicity of human V
segments.

A 6.3 kb BamHI/HindIII fragment containing human J
segments (see fragment (a) in Fig. 9) is cloned into MluI/SpeI
digested pHIG5' using the following adapters:

15

                    5' GAT CCA AGC AGT 3'

                    5' CTA GAC TGC TTG 3'

                    5' CGC GTC GAA CTA 3'
20
                    5' AGC TTA GTT CGA 3'

The resultant is plasmid designated pHIG5'O
(overlap). The insert contained in this plasmid contains
25 human V, D and J segments. When the single V segment from
pVH1 is used, the size of this insert is approximately 17 kb
plus 2 kb. This insert is isolated and combined with the
insert from pHIG3' which contains the human J, Cμ, γ1 and rat
3' enhancer sequences. Both inserts contain human J segments
30 which provide for approximately 6.3 kb of overlap between the
two DNA fragments. When coinjected into the mouse zygote, in
vivo homologous recombination occurs generating a transgene
equivalent to the insert contained in pHIG.

This approach provides for the addition of a multiplicity of V segments into the transgene formed in vivo. For example, instead of incorporating a single V segment into pHIG5', a multiplicity of V segments contained on (1) isolated genomic DNA, (2) ligated DNA derived from genomic DNA, or (3) DNA encoding a synthetic V segment repertoire is cloned into pHIG2 at the SfiI site to generate pHIG5' V$_N$. The J segments fragment (a) of Fig. 9 is then cloned into pHIG5' V$_N$ and the insert isolated. This insert now contains a multiplicity of V segments and J segments which overlap with the J segments contained on the insert isolated from pHIG3'. When cointroduced into the nucleus of a mouse zygote, homologous recombination occurs to generate in vivo the transgene encoding multiple V segments and multiple J segments, multiple D segments, the Cμ region, the Cγ1 region (all from human) and the rat 3' enhancer sequence.

EXAMPLE 5

Construction of Light Chain Minilocus

A. **Construction of pEμ1**

The construction of pEμ1 is depicted in Fig. 16. The mouse heavy chain enhancer is isolated on the XbaI to EcoRI 678 bp fragment (Banerji et al., Cell 33:729-740 (1983)) from phage clones using oligo:

5' GAA TGG GAG TGA GGC TCT CTC ATA CCC
     TAT TCA GAA CTG ACT 3'

This Eμ fragment is cloned into EcoRV/XbaI digested pGP1 by blunt end filling in EcoRI site. The resultant plasmid is designated pEmu1.

B. <u>Construction Of κ Light chain Minilocus</u>

The κ construct contains at least one human $V_κ$ segment, all five human $J_κ$ segments, the human $J-C_κ$ enhancer, human κ constant region exon, and, ideally, the human 3' κ enhancer (Meyer et al., <u>EMBO J.</u> <u>8</u>:1959-1964 (1989)). The κ enhancer in mouse is 9 kb downstream from $C_κ$. However, it is as yet unidentified in the human. In addition, the construct contains a copy of the mouse heavy chain $J-C\mu$ enhancers.

The minilocus is constructed from four component fragments:

(a) A 16 kb SmaI fragment that contains the human $C_κ$ exon and the 3' human enhancer by analogy with the mouse locus;

(b) A 5' adjacent 5 kb SmaI fragment, which contains all five J segments;

(c) The mouse heavy chain intronic enhancer isolated from pEμ1 (this sequence is included to induce expression of the light chain construct as early as possible in B-cell development. Because the heavy chain genes are transcribed earlier than the light chain genes, this heavy chain enhancer is presumably active at an earlier stage than the intronic κ enhancer); and

(d) A fragment containing one or more V segments.

The preparation of this construct is as follows. Human placental DNA is digested with SmaI and fractionated on agarose gel by electrophoresis. Similarly, human placental DNA is digested with BamHI and fractionated by electrophoresis. The 16 kb fraction is isolated from the SmaI digested gel and the 11 kb region is similarly isolated from the gel containing DNA digested with BamHI.

The 16 kb SmaI fraction is cloned into Lambda FIX II (Stratagene, La Jolla, California) which has been digested

with XhoI, treated with klenow fragment DNA polymerase to fill
in the XhoI restriction digest product. Ligation of the 16 kb
SmaI fraction destroys the SmaI sites and lases XhoI sites
intact.

5   The 11 kb BamHI fraction is cloned into λ EMBL3
(Strategene, La Jolla, California) which is digested with
BamHI prior to cloning.

         Clones from each library were probed with the Cκ
specific oligo:

10

                5' GAA CTG TGG CTG CAC CAT CTG TCT
                   TCA TCT TCC CGC CAT CTG 3'

15   A 16 kb XhoI insert that was subcloned into the XhoI
cut pEμ1 so that Cκ is adjacent to the SmaI site. The
resultant plasmid was designated pKap1.

         The above Cκ specific oligonucleotide is used to
probe the λ EMBL3/BamHI library to identify an 11 kb clone. A
20 5 kb SmaI fragment (fragment (b) in Fig. 20) is subcloned and
subsequently inserted into pKap1 digested with SmaI. Those
plasmids containing the correct orientation of J segments, Cκ
and the Eμ enhancer are designated pKap2.

         One or more Vκ segments are thereafter subcloned
25 into the MluI site of pKap2 to yield the plasmid pKapH which
encodes the human Vκ segments, the human Jκ segments, the
human Cκ segments and the human Eμ enhancer. This insert is
excised by digesting pKapH with NotI and purified by agarose
gel electrophoresis. The thus purified insert is
30 microinjected into the pronucleus of a mouse zygote as
previously described.

C.   Construction of κ Light Chain Minilocus by
      In Vivo Homologous Recombination

The 11 kb BamHI fragment is cloned into BamHI
digested pGP1 such that the 3' end is toward the SfiI site.
The resultant plasmid is designated pKAPint. One or more Vκ
segments is inserted into the polylinker between the BamHI and

5   SpeI sites in pKAPint to form pKapHV. The insert of pKapHV is
excised by digestion with NotI and purified. The insert from
pKap2 is excised by digestion with NotI and purified. Each of
these fragments contain regions of homology in that the
fragment from pKapHV contains a 5 kb sequence of DNA that

10   include the J_κ segments which is substantially homologous to
the 5 kb SmaI fragment contained in the insert obtained from
pKap2. As such, these inserts are capable of homologously
recombining when microinjected into a mouse zygote to form a
transgene encoding V_κ, J_κ and C_κ.

15

EXAMPLE 6

Isolation of Genomic Clones
Corresponding to Rearranged and Expressed
20   Copies of Immunoglobulin κ Light Chain Genes

This example describes the cloning of immunoglobulin
κ light chain genes from cultured cells that express an
immunoglobulin of interest. Such cells may contain multiple
25   alleles of a given immunoglobulin gene. For example, a
hybridoma might contain four copies of the κ light chain gene,
two copies from the fusion partner cell line and two copies
from the original B-cell expressing the immunoglobulin of
interest. Of these four copies, only one encodes the
30   immunoglobulin of interest, despite the fact that several of
them may be rearranged. The procedure described in this
example allows for the selective cloning of the expressed copy
of the κ light chain.

A.   Double Stranded cDNA

        Cells from human hybridoma, or lymphoma, or other
cell line that synthesizes either cell surface or secreted or
both forms of IgM with a κ light chain are used for the
5   isolation of polyA+ RNA. The RNA is then used for the
synthesis of oligo dT primed cDNA using the enzyme reverse
transcriptase (for general methods see, Goodspeed et al.
(1989) Gene 76: 1; Dunn et al. (1989) J. Biol. Chem. 264:
13057). The single stranded cDNA is then isolated and G
10   residues are added to the 3' end using the enzyme
polynucleotide terminal transferase. The G-tailed
single-stranded cDNA is then purified and used as template for
second strand synthesis (catalyzed by the enzyme DNA
polymerase) using the following oligonucleotide as a primer:

15

                5' - GAG GTA CAC TGA CAT ACT GGC ATG CCC
                          CCC CCC CCC - 3'

20   The double stranded cDNA is isolated and used for
determining the nucleotide sequence of the 5' end of the mRNAs
encoding the heavy and light chains of the expressed
immunoglobulin molecule. Genomic clones of these expressed
genes are then isolated. The procedure for cloning the
25   expressed light chain gene is outlined in part B below.

B.   Light Chain

        The double stranded cDNA described in part A is
denatured and used as a template for a third round of DNA
30   synthesis using the following oligonucleotide primer:

        5' - GTA CGC CAT ATC AGC TGG ATG AAG TCA TCA GAT
                GGC GGG AAG ATG AAG ACA GAT GGT GCA - 3'

35

This primer contains sequences specific for the
constant portion of the κ light chain message (TCA TCA GAT GGC
GGG AAG ATG AAG ACA GAT GGT GCA) as well as unique sequences
that can be used as a primer for the PCR amplification of the
5 newly synthesized DNA strand (GTA CGC CAT ATC AGC TGG ATG
AAG). The sequence is amplified by PCR using the following
two oligonucleotide primers:

                     5' - GAG GTA CAC TGA CAT ACT GGC ATG -3'
10   5' - GTA CGC CAT ATC AGC TGG ATG AAG -3'

     The PCR amplified sequence is then purified by gel
electrophoresis and used as template for dideoxy sequencing
15 reactions using the following oligonucleotide as a primer:

          5' - GAG GTA CAC TGA CAT ACT GGC ATG -3'

     The first 42 nucleotides of sequence will then be
20 used to synthesize a unique probe for isolating the gene from
which immunoglobulin message was transcribed. This synthetic
42 nucleotide segment of DNA will be referred to below as
o-kappa.
     A Southern blot of DNA, isolated from the Ig
25 expressing cell line and digested individually and in pairwise
combinations with several different restriction endonucleases
including SmaI, is then probed with the 32-P labelled unique
oligonucleotide o-kappa. A unique restriction endonuclease
site is identified upstream of the rearranged V segment.
30   DNA from the Ig expressing cell line is then cut
with SmaI and second enzyme (or BamHI or KpnI if there is SmaI
site inside V segment). Any resulting non-blunted ends are
treated with the enzyme T4 DNA polymerase to give blunt ended

DNA molecules. Then add restriction site encoding linkers
(BamHI, EcoRI or XhoI depending on what site does not exist in
fragment) and cut with the corresponding linker enzyme to give
DNA fragments with BamHI, EcoRI or XhoI ends. The DNA is then
5   size fractionated by agarose gel electrophoresis, and the
fraction including the DNA fragment covering the expressed V
segment is cloned into lambda EMBL3 or Lambda FIX (Stratagene,
La Jolla, California). V segment containing clones are
isolated using the unique probe o-kappa. DNA is isolated from
10 positive clones and subcloned into the polylinker of pKap1.
The resulting clone is called pRKL.

EXAMPLE 7

Isolation of Genomic Clones
15   Corresponding to Rearranged Expressed Copies
of Immunoglobulin Heavy Chain μ Genes

        This example describes the cloning of immunoglobulin
heavy chain μ genes from cultured cells of expressed and
immunoglobulin of interest. The procedure described in this
20 example allows for the selective cloning of the expressed copy
of a μ heavy chain gene.

        Double-stranded cDNA is prepared and isolated as
described herein before. The double-stranded cDNA is
denatured and used as a template for a third round of DNA
25 synthesis using the following oligonucleotide primer:

        5' - GTA CGC CAT ATC AGC TGG ATG AAG ACA GGA GAC
                    GAG GGG GAA AAG GGT TGG GGC GGA TGC - 3'

30   This primer contains sequences specific for the
constant portion of the μ heavy chain message (ACA GGA GAC GAG
GGG GAA AAG GGT TGG GGC GGA TGC) as well as unique sequences

that can be used as a primer for the PCR amplification of the
newly synthesized DNA strand (GTA CGC CAT ATC AGC TGG ATG
AAG). The sequence is amplified by PCR using the following
two oligonucleotide primers:

5

5' - GAG GTA CAC TGA CAT ACT GGC ATG - 3'
5' - GTA CTC CAT ATC AGC TGG ATG AAG - 3'

The PCR amplified sequence is then purified by gel
10 electrophoresis and used as template for dideoxy sequencing
reactions using the following oligonucleotide as a primer:

5' - GAG GTA CAC TGA CAT ACT GGC ATG - 3'

15   The first 42 nucleotides of sequence are then used
to synthesize a unique probe for isolating the gene from
which immunoglobulin message was transcribed. This synthetic
42 nucleotide segment of DNA will be referred to below as
o-mu.

20   A Southern blot of DNA, isolated from the Ig
expressing cell line and digested individually and in pairwise
combinations with several different restriction endonucleases
including MluI (MluI is a rare cutting enzyme that cleaves
between the J segment and mu CH1), is then probed with the
25 32-P labelled unique oligonucleotide o-mu. A unique
restriction endonuclease site is identified upstream of the
rearranged V segment.

DNA from the Ig expressing cell line is then cut
with MluI and second enzyme. MluI or SpeI adapter linkers are
30 then ligated onto the ends and cut to convert the upstream
site to MluI or SpeI. The DNA is then size fractionated by
agarose gel electrophoresis, and the fraction including the

DNA fragment covering the expressed V segment is cloned
directly into the plasmid pGPI. V segment containing clones
are isolated using the unique probe o-mu, and the insert is
subcloned into MluI or MluI/SpeI cut plasmid pCON2. The
5   resulting plasmid is called pRMGH.

EXAMPLE 8

Construction of Human κ Miniloci Transgenes
Light Chain Minilocus
10   A human genomic DNA phage library was screened with
kappa light chain specific oligonucleotide probes and isolated
clones spanning the J_κ-C region. A 5.7 kb ClaI/XhoI fragment
containing J_κ1 together with a 13 kb XhoI fragment containing
J_κ2-5 and C_κ into pGP1d was cloned and used to create the
15 plasmid pKcor. This plasmid contains J_κ1-5, the kappa intronic
enhancer and C_κ together with 4.5 kb of 5' and 9 kb of 3'
flanking sequences. It also has a unique 5' XhoI site for
cloning V_κ segments and a unique 3' SalI site for inserting
additional cis-acting regulatory sequences.
20

V kappa genes
          A human genomic DNA phage library was screened with
V_κ light chain specific oligonucleotide probes and isolated
clones containing human V_κ segments. Functional V segments
25 were identified by DNA sequence analysis. These clones
contain TATA boxes, open reading frames encoding leader and
variable peptides (including 2 cysteine residues), splice
sequences, and recombination heptamer-12 bp spacer-nonamer
sequences. Three of the clones were mapped and sequenced.
30 Two of the clones, 65.5 and 65.8 appear to be functional, they
contain TATA boxes, open reading frames encoding leader and
variable peptides (including 2 cysteine residues), splice

sequences, and recombination heptamer-12 bp spacer-nonamer
sequences. The third clone, 65.4, appears to encode a V_kI
pseudogene as it contains a non-canonical recombination
heptamer.

5   One of the functional clones, Vk 65-8, which encodes
a VkIII family gene, was used to build a light chain minilocus
construct.

<u>pKC1</u>

10   The kappa light chain minilocus transgene pKC1 (Fig.
32) was generated by inserting a 7.5 kb XhoI/SalI fragment
containing V_k 65.8 into the 5' XhoI site of pKcor. The
transgene insert was isolated by digestion with NotI prior to
injection.

15   The purified insert was microinjected into the
pronuclei of fertilized (C57BL/6 x CBA)F2 mouse embryos and
transferred the surviving embryos into pseudopregnant females
as described by Hogan et al. (<u>in</u> Methods of Manipulating the
Mouse Embryo, 1986, Cold Spring Harbor Laboratory, New York).

20 Mice that developed from injected embryos were analyzed for
the presence of transgene sequences by Southern blot analysis
of tail DNA. Transgene copy number was estimated by band
intensity relative to control standards containing known
quantities of cloned DNA. Serum was isolated from these

25 animals and assayed for the presence of transgene encoded
human Ig kappa protein by ELISA as described by Harlow and
Lane (<u>in</u> Antibodies: A Laboratory Manual, 1988, Cold Spring
Harbor Laboratory, New York). Microtiter plate wells were
coated with mouse monoclonal antibodies specific for human Ig

30 kappa (clone 6E1, #0173, AMAC, Inc., Westbrook, ME), human IgM
(Clone AF6, #0285, AMAC, Inc., Westbrook, ME) and human IgG1
(clone JL512, #0280, AMAC, Inc., Westbrook, ME). Serum

samples were serially diluted into the wells and the presence
of specific immunoglobulins detected with affinity isolated
alkaline phosphatase conjugated goat anti-human Ig
(polyvalent) that had been pre-adsorbed to minimize cross-
5   reactivity with mouse immunoglobulins.

Fig. 35 shows the results of an ELISA assay of serum
from 8 mice (I.D. #676, 674, 673, 670, 666, 665, 664, and
496). The first seven of these mice developed from embryos
that were injected with the pKC1 transgene insert and the
10 eighth mouse is derived from a mouse generated by
microinjection of the pHC1 transgene (described previously).
Two of the seven mice from KC1 injected embryos (I.D.#'s 666
and 664) did not contain the transgene insert as assayed by
DAN Southern blot analysis, and five of the mice (I.D.#'s 676,
15 674, 673, 670, and 665) contained the transgene. All but one
of the KC1 transgene positive animals express detectable
levels of human Ig kappa protein, and the single non-
expressing animal appears to be a genetic mosaic on the basis
of DNA Southern blot analysis. The pHC1 positive transgenic
20 mouse expresses human IgM and IgG1 but not Ig kappa,
demonstrating the specificity of the reagents used in the
assay.

pKC2

25   The kappa light chain minilocus transgene pKC2 was
generated by inserting an 8 kb XhoI/SalI fragment containing
V_K 65.5 into the 5' XhoI site of pKC1. The resulting
transgene insert, which contains two V_K segments, was isolated
prior to microinjection by digestion with NotI.

30

pKVe2

This construct is identical to pKC1 except that it
includes 1.2 kb of additional sequence 5' of J_κ and is missing
4.5 kb of sequence 3' of V_κ 65.8. In additional it contains a
0.9 kb XbaI fragment containing the mouse heavy chain J-μ

5   intronic enhancer (Banerji et al., Cell 33:729-740 (1983))
together with a 1.4 kb MluI/HindIII fragment containing the
human heavy chain J-μ intronic enhancer (Hayday et al., Nature
307:334-340 (1984)) inserted downstream. This construct tests
the feasibility of initiating early rearrangement of the light

10   chain minilocus to effect allelic and isotypic exclusion.
Analogous constructs can be generated with different
enhancers, i.e., the mouse or rat 3' kappa or heavy chain
enhancer (Meyer and Neuberger, EMBO J. 8:1959-1964 (1989);
Petterson et al. Nature 344:165-168 (1990), which are

15   incorporated herein by reference).

Rearranged Light Chain Transgenes

A kappa light chain expression cassette was designed
to reconstruct functionally rearranged light chain genes that

20   have been amplified by PCR from human B-cell DNA. The scheme
is outlined in Fig. 33. PCR amplified light chain genes are
cloned into the vector pK5nx that includes 3.7 kb of 5'
flanking sequences isolated from the kappa light chain gene
65.5. The VJ segment fused to the 5' transcriptional

25   sequences are then cloned into the unique XhoI site of the
vector pK31s that includes J_κ2-4, the J_κ intronic enhancer, C_κ,
and 9 kb of downstream sequences. The resulting plasmid
contains a reconstructed functionally rearranged kappa light
chain transgene that can be excised with NotI for

30   microinjection into embryos. The plasmids also contain unique
SalI sites at the 3' end for the insertion of additional cis-
acting regulatory sequences.

Two synthetic oligonucleotides (o-130, o-131) were used to amplify rearranged kappa light chain genes from human spleen genomic DNA. Oligonucleotide o-131 (gga ccc aga (g,c)gg aac cat gga a(g,a)(g,a,t,c)) is complementary to the

5   5' region of $V_\kappa III$ family light chain genes and overlaps the first ATC of the leader sequence. Oligonucleotide o-130 (gtg caa tca att ctc gag ttt gac tac aga c) is complementary to a sequence approximately 150 bp 3' of $J_\kappa 1$ and includes an XhoI site. These two oligonucleotides amplify a 0.7 kb DNA

10 fragment from human spleen DNA corresponding to rearranged $V_\kappa III$ genes joined to $J_\kappa 1$ segments. The PCR amplified DNA was digested with NcoI and XhoI and cloned individual PCR products into the plasmid pNN03. The DNA sequence of 5 clones was determined and identified two with functional VJ joints (open

15 reading frames). Additional functionally rearranged light chain clones are collected. The functionally rearranged clones can be individually cloned into light chain expression cassette described above (Fig. 33). Transgenic mice generated with the rearranged light chain constructs can be bred with

20 heavy chain minilocus transgenics to produce a strain of mice that express a spectrum of fully human antibodies in which all of the diversity of the primary repertoire is contributed by the heavy chain. One source of light chain diversity can be from somatic mutation. Because not all light chains will be

25 equivalent with respect to their ability to combine with a variety of different heavy chains, different strains of mice, each containing different light chain constructs can be generated and tested. The advantage of this scheme, as opposed to the use of unrearranged light chain miniloci, is

30 the increased light chain allelic and isotypic exclusion that comes from having the light chain ready to pair with a heavy chain as soon as heavy chain VDJ joining occurs. This

combination can result in an increased frequency of B-cells
expressing fully human antibodies, and thus it can facilitate
the isolation of human Ig expressing hybridomas.

5   NotI inserts of plasmids pIGM1, pHC1, pIGG1, pKC1,
and pKC2 were isolated away from vector sequences by agarose
gel electrophoresis. The purified inserts were microinjected
into the pronuclei of fertilized (C57BL/6 x CBA)F2 mouse
embryos and transferred the surviving embryos into
pseudopregnant females as described by Hogan et al. (Hogan et
10   al., Methods of Manipulating the Mouse Embryo, Cold Spring
Harbor Laboratory, New York (1986)).

EXAMPLE 9

Inactivation of the Mouse Kappa Light Chain Gene by Homologous
15   Recombination

This example describes the inactivation of the mouse
endogenous kappa locus by homologous recombination in
embryonic stem (ES) cells followed by introduction of the
20   mutated gene into the mouse germ line by injection of targeted
ES cells bearing an inactivated kappa allele into early mouse
embryos (blastocysts).

The strategy is to delete J_κ and C_κ by homologous
recombination with a vector containing DNA sequences
25   homologous to the mouse kappa locus in which a 4.5 kb segment
of the locus, spanning the J_κ gene and C_κ segments, is deleted
and replaced by the selectable marker neo.

Construction of the kappa targeting vector

30   The plasmid pGEM7 (KJ1) contains the neomycin
resistance gene (neo), used for drug selection of transfected
ES cells, under the transcriptional control of the mouse
phosphoglycerate kinase (pgk) promoter (XbaI/TaqI fragment;

Adra et al. (1987) Gene 60: 65) in the cloning vector pGEM-7Zf(+). The plasmid also includes a heterologous polyadenylation site for the neo gene, derived from the 3' region of the mouse pgk gene (PvuII/HindIII fragment; Boer et

5 al., Biochemical Genetics, 28:299-308 (1990)). This plasmid was used as the starting point for construction of the kappa targeting vector. The first step was to insert sequences homologous to the kappa locus 3' of the neo expression cassette.

10   Mouse kappa chain sequences (Fig. 20a) were isolated from a genomic phage library derived from liver DNA using oligonucleotide probes specific for the Cκ locus:

5'- GGC TGA TGC TGC ACC AAC TGT ATC CAT CTT CCC ACC ATC CAG

15   -3'

and for the Jκ5 gene segment:

5'- CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGT AAG -

20   3'.

      An 8 kb BglII/SacI fragment extending 3' of the mouse C_K segment was isolated from a positive phage clone in two pieces, as a 1.2 kb BglII/SacI fragment and a 6.8 kb SacI fragment, and subcloned into BglII/SacI digested pGEM7 (KJ1)

25 to generate the plasmid pNEO-K3' (Fig. 20b).

      A 1.2 kb EcoRI/SphI fragment extending 5' of the J_K region was also isolated from a positive phage clone. An SphI/XbaI/BglII/EcoRI adaptor was ligated to the SphI site of this fragment, and the resulting EcoRI fragment was ligated

30 into EcoRI digested pNEO-K3', in the same 5' to 3' orientation as the neo gene and the downstream 3' kappa sequences, to generate pNEO-K5'3' (Fig. 20c).

The Herpes Simplex Virus (HSV) thymidine kinase (TK)
gene was then included in the construct in order to allow for
enrichment of ES clones bearing homologous recombinants, as
described by Mansour et al., Nature 336:348-352 (1988), which

5 is incorporated herein by reference. The HSV TK cassette was
obtained from the plasmid pGEM7 (TK), which contains the
structural sequences for the HSV TK gene bracketed by the
mouse pgk promoter and polyadenylation sequences as described
above for pGEM7 (KJ1). The EcoRI site of pGEM7 (TK) was

10 modified to a BamHI site and the TK cassette was then excised
as a BamHI/HindIII fragment and subcloned into pGP1b to
generate pGP1b-TK. This plasmid was linearized at the XhoI
site and the XhoI fragment from pNEO-K5'3', containing the neo
gene flanked by genomic sequences from 5' of Jĸ and 3' of Cĸ,

15 was inserted into pGP1b-TK to generate the targeting vector
J/C KI (Fig. 20d). The putative structure of the genomic
kappa locus following homologous recombination with J/C K1 is
shown in Fig. 20e.

20 Generation and analysis of ES cells with targeted inactivation
of a kappa allele
The ES cells used were the AB-1 line grown on
mitotically inactive SNL76/7 cell feeder layers (McMahon and
Bradley, Cell 62:1073-1085 (1990)) essentially as described

25 (Robertson, E.J. (1987) in Teratocarcinomas and Embryonic Stem
Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL
Press), p. 71-112). Other suitable ES lines include, but are
not limited to, the E14 line (Hooper et al. (1987) Nature 326:
292-295), the D3 line (Doetschman et al. (1985) J. Embryol.

30 Exp. Morph. 87: 27-45), and the CCE line (Robertson et al.
(1986) Nature 323: 445-448). The success of generating a
mouse line from ES cells bearing a specific targeted mutation

depends on the pluripotence of the ES cells (i.e., their
ability, once injected into a host blastocyst, to participate
in embryogenesis and contribute to the germ cells of the
resulting animal).

5   The pluripotence of any given ES cell line can vary
with time in culture and the care with which it has been
handled. The only definitive assay for pluripotence is to
determine whether the specific population of ES cells to be
used for targeting can give rise to chimeras capable of
10 germline transmission of the ES genome. For this reason,
prior to gene targeting, a portion of the parental population
of AB-1 cells is injected into C57Bl/6J blastocysts to
ascertain whether the cells are capable of generating chimeric
mice with extensive ES cell contribution and whether the
15 majority of these chimeras can transmit the ES genome to
progeny.

        The kappa chain inactivation vector J/C K1 was
digested with NotI and electroporated into AB-1 cells by the
methods described (Hasty et al., Nature, 350:243-246 (1991)).
20 Electroporated cells were plated onto 100 mm dishes at a
density of 1-2 x 10^6 cells/dish. After 24 hours, G418
(200μg/ml of active component) and FIAU (0.5μM) were added to
the medium, and drug-resistant clones were allowed to develop
over 10-11 days. Clones were picked, trypsinized, divided
25 into two portions, and further expanded. Half of the cells
derived from each clone were then frozen and the other half
analyzed for homologous recombination between vector and
target sequences.

        DNA analysis was carried out by Southern blot
30 hybridization. DNA was isolated from the clones as described
(Laird et al., Nucl. Acids Res. 19:4293 (1991)) digested with
XbaI and probed with the 800 bp EcoRI/XbaI fragment indicated

in Fig. 20e as probe A. This probe detects a 3.7 kb XbaI
fragment in the wild type locus, and a diagnostic 1.8 kb band
in a locus which has homologously recombined with the
targeting vector (see Fig. 20a and e). Of 901 G418 and FIAU

5   resistant clones screened by Southern blot analysis, 7
displayed the 1.8 kb XbaI band indicative of a homologous
recombination into one of the kappa genes. These 7 clones
were further digested with the enzymes BglII, SacI, and PstI
to verify that the vector integrated homologously into one of

10 the kappa genes. When probed with the diagnostic 800 bp
EcoRI/XbaI fragment (probe A), BglII, SacI, and PstI digests
of wild type DNA produce fragments of 4.1, 5.4, and 7 kb,
respectively, whereas the presence of a targeted kappa allele
would be indicated by fragments of 2.4, 7.5, and 5.7 kb,

15 respectively (see Fig. 20a and e). All 7 positive clones
detected by the XbaI digest showed the expected BglII, SacI,
and PstI restriction fragments diagnostic of a homologous
recombination at the kappa light chain. In addition, Southern
blot analysis of an NsiI digest of the targeted clones using a

20 neo specific probe (probe B, Fig. 20e) generated only the
predicted fragment of 4.2 kb, demonstrating that the clones
each contained only a single copy of the targeting vector.

Generation of mice bearing the inactivated kappa chain

25   Five of the targeted ES clones described in the
previous section were thawed and injected into C57Bl/6J
blastocysts as described (Bradley, A. (1987) in
Teratocarcinomas and Embryonic Stem Cells: A Practical
Approach. E.J. Robertson, ed. (Oxford: IRL Press), p. 113-151)

30 and transferred into the uteri of pseudopregnant females to
generate chimeric mice resulting from a mixture of cells
derived from the input ES cells and the host blastocyst. The

extent of ES cell contribution to the chimeras can be visually estimated by the amount of agouti coat coloration, derived from the ES cell line, on the black C57Bl/6J background. Approximately half of the offspring resulting from blastocyst

5 injection of the targeted clones were chimeric (i.e., showed agouti as well as black pigmentation) and of these, the majority showed extensive (70 percent or greater) ES cell contribution to coat pigmentation. The AB1 ES cells are an XY cell line and a majority of these high percentage chimeras

10 were male due to sex conversion of female embryos colonized by male ES cells. Male chimeras derived from 4 of the 5 targeted clones were bred with C57BL/6J females and the offspring monitored for the presence of the dominant agouti coat color indicative of germline transmission of the ES genome.

15 Chimeras from two of these clones consistently generated agouti offspring. Since only one copy of the kappa locus was targeted in the injected ES clones, each agouti pup had a 50 percent chance of inheriting the mutated locus. Screening for the targeted gene was carried out by Southern blot analysis of

20 Bgl II-digested DNA from tail biopsies, using the probe utilized in identifying targeted ES clones (probe A, Fig. 20e). As expected, approximately 50 percent of the agouti offspring showed a hybridizing Bgl II band of 2.4 kb in addition to the wild-type band of 4.1 kb, demonstrating the

25 germline transmission of the targeted kappa locus.

In order to generate mice homozygous for the mutation, heterozygotes were bred together and the kappa genotype of the offspring determined as described above. As expected, three genotypes were derived from the heterozygote

30 matings: wild-type mice bearing two copies of a normal kappa locus, heterozygotes carrying one targeted copy of the kappa gene and one NT kappa gene, and mice homozygous for the kappa

mutation. The deletion of kappa sequences from these latter

mice was verified by hybridization of the Southern blots with

a probe specific for J_κ (probe C, Fig. 20a). Whereas

hybridization of the J_κ probe was observed to DNA samples from

5   heterozygous and wild-type siblings, no hybridizing signal was

present in the homozygotes, attesting to the generation of a

novel mouse strain in which both copies of the kappa locus

have been inactivated by deletion as a result of targeted

mutation.

10

EXAMPLE 10

Inactivation of the Mouse Heavy Chain Gene by Homologous

Recombination

This example describes the inactivation of the

15   endogenous murine immunoglobulin heavy chain locus by

homologous recombination in embryonic stem (ES) cells. The

strategy is to delete the endogenous heavy chain J segments by

homologous recombination with a vector containing heavy chain

sequences from which the J_H region has been deleted and

20   replaced by the gene for the selectable marker neo.

Construction of a heavy chain targeting vector

Mouse heavy chain sequences containing the J_H region

(Fig. 21a) were isolated from a genomic phage library derived

25   from the D3 ES cell line (Gossler et al., Proc. Natl. Acad.

Sci. U.S.A. 83:9065-9069 (1986)) using a J_H4 specific

oligonucleotide probe:

5'- ACT ATG CTA TGG ACT ACT GGG GTC AAG GAA CCT CAG TCA CCG

30   -3'

A 3.5 kb genomic SacI/StuI fragment, spanning the J_H

region, was isolated from a positive phage clone and subcloned

into SacI/SmaI digested pUC18. The resulting plasmid was designated pUC18 J$_H$. The neomycin resistance gene (neo), used for drug selection of transfected ES cells, was derived from a repaired version of the plasmid pGEM7 (KJ1). A report in the literature (Yenofsky et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 3435-3439) documents a point mutation the neo coding sequences of several commonly used expression vectors, including the construct pMC1neo (Thomas and Cappechi (1987) Cell 51: 503-512) which served as the source of the neo gene used in pGEM7 (KJ1). This mutation reduces the activity of the neo gene product and was repaired by replacing a restriction fragment encompassing the mutation with the corresponding sequence from a wild-type neo clone. The HindIII site in the prepared pGEM7 (KJ1) was converted to a SalI site by addition of a synthetic adaptor, and the neo expression cassette excised by digestion with XbaI/SalI. The ends of the neo fragment were then blunted by treatment with the Klenow form of DNA polI, and the neo fragment was subcloned into the NaeI site of pUC18 J$_H$, generating the plasmid pUC18 J$_H$-neo (Fig. 21b).

Further construction of the targeting vector was carried out in a derivative of the plasmid pGP1b. pGP1b was digested with the restriction enzyme NotI and ligated with the following oligonucleotide as an adaptor:

5'- GGC CGC TCG ACG ATA GCC TCG AGG CTA TAA ATC TAG AAG AAT TCC AGC AAA GCT TTG GC -3'

The resulting plasmid, called pGMT, was used to build the mouse immunoglobulin heavy chain targeting construct.

The Herpes Simplex Virus (HSV) thymidine kinase (TK) gene was included in the construct in order to allow for enrichment of ES clones bearing homologous recombinants, as described by Mansour et al. (Nature 336, 348-352 (1988)). The

5 HSV TK gene was obtained from the plasmid pGEM7 (TK) by digestion with EcoRI and HindIII. The TK DNA fragment was subcloned between the EcoRI and HindIII sites of pGMT, creating the plasmid pGMT-TK (Fig. 21c).

To provide an extensive region of homology to the

10 target sequence, a 5.9 kb genomic XbaI/XhoI fragment, situated 5' of the J_H region, was derived from a positive genomic phage clone by limit digestion of the DNA with XhoI, and partial digestion with XbaI. As noted in Fig. 21a, this XbaI site is not present in genomic DNA, but is rather derived from phage

15 sequences immediately flanking the cloned genomic heavy chain insert in the positive phage clone. The fragment was subcloned into XbaI/XhoI digested pGMT-TK, to generate the plasmid pGMT-TK-J_H5' (Fig. 21d).

The final step in the construction involved the

20 excision from pUC18 J_H-neo of the 2.8 kb EcoRI fragment which contained the neo gene and flanking genomic sequences 3' of J_H. This fragment was blunted by Klenow polymerase and subcloned into the similarly blunted XhoI site of pGMT-TK-J_H5'. The resulting construct, J_HKO1 (Fig. 21e), contains 6.9 kb of

25 genomic sequences flanking the J_H locus, with a 2.3 kb deletion spanning the J_H region into which has been inserted the neo gene. Fig. 21f shows the structure of an endogenous heavy chain gene after homologous recombination with the targeting construct.

30

EXAMPLE 11

Generation and analysis of targeted ES cells

AB-1 ES cells (McMahon and Bradley, Cell
62:1073-1085 (1990)) were grown on mitotically inactive
5 SNL76/7 cell feeder layers essentially as described
(Robertson, E.J. (1987) Teratocarcinomas and Embryonic Stem
Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL
Press), pp. 71-112). As described in the previous example,
prior to electroporation of ES cells with the targeting
10 construct J$_H$KO1, the pluripotency of the ES cells was
determined by generation of AB-1 derived chimeras which were
shown capable of germline transmission of the ES genome.

The heavy chain inactivation vector J$_H$KO1 was
digested with NotI and electroporated into AB-1 cells by the
15 methods described (Hasty et al., Nature 350:243-246 (1991)).
Electroporated cells were plated into 100 mm dishes at a
density of 1-2 x 10^6 cells/dish. After 24 hours, G418
(200mg/ml of active component) and FIAU (0.5mM) were added to
the medium, and drug-resistant clones were allowed to develop
20 over 8-10 days. Clones were picked, trypsinized, divided into
two portions, and further expanded. Half of the cells derived
from each clone were then frozen and the other half analyzed
for homologous recombination between vector and target
sequences.

25 DNA analysis was carried out by Southern blot
hybridization. DNA was isolated from the clones as described
(Laird et al. (1991) Nucleic Acids Res. 19: 4293), digested
with StuI and probed with the 500 bp EcoRI/StuI fragment
designated as probe A in Fig. 21f. This probe detects a StuI
30 fragment of 4.7 kb in the wild-type locus, whereas a 3 kb band
is diagnostic of homologous recombination of endogenous
sequences with the targeting vector (see Fig. 21a and f). Of

525 G418 and FIAU doubly-resistant clones screened by Southern

blot hybridization, 12 were found to contain the 3 kb fragment

diagnostic of recombination with the targeting vector. That

these clones represent the expected targeted events at the J_H

5   locus (as shown in Fig. 21f) was confirmed by further

digestion with HindIII, SpeI and HpaI. Hybridization of probe

A (see Fig. 21f) to Southern blots of HindIII, SpeI, and HpaI

digested DNA produces bands of 2.3 kb, >10 kb, and >10kb,

respectively, for the wild-type locus (see Fig. 21a), whereas

10 bands of 5.3 kb, 3.8 kb, and 1.9 kb, respectively, are

expected for the targeted heavy chain locus (see Fig 21f).

All 12 positive clones detected by the StuI digest showed the

predicted HindIII, SpeI, and HpaI bands diagnostic of a

targeted J_H gene. In addition, Southern blot analysis of a

15 StuI digest of all 12 clones using a neo-specific probe (probe

B, Fig. 21f) generated only the predicted fragment of 3 kb,

demonstrating that the clones each contained only a single

copy of the targeting vector.

20 Generation of mice carrying the J_H deletion

            Three of the targeted ES clones described in the

previous section were thawed and injected into C57BL/6J

blastocysts as described (Bradley, A. (1987) in

Teratocarcinomas and Embryonic Stem Cells: A Practical

25 Approach, E.J. Robertson, ed. (Oxford: IRL Press), p.113-151)

and transferred into the uteri of pseudopregnant females. The

extent of ES cell contribution to the chimera was visually

estimated from the amount of agouti coat coloration, derived

from the ES cell line, on the black C57BL/6J background. Half

30 of the offspring resulting from blastocyst injection of two of

the targeted clones were chimeric (i.e., showed agouti as well

as black pigmentation); the third targeted clone did not

generate any chimeric animals. The majority of the chimeras

showed significant (approximately 50 percent or greater) ES

cell contribution to coat pigmentation. Since the AB-1 ES

cells are an XY cell line, most of the chimeras were male, due

5   to sex conversion of female embryos colonized by male ES

cells. Males chimeras were bred with C57BL/6J females and the

offspring monitored for the presence of the dominant agouti

coat color indicative of germline transmission of the ES

genome. Chimeras from both of the clones consistently

10   generated agouti offspring. Since only one copy of the heavy

chain locus was targeted in the injected ES clones, each

agouti pup had a 50 percent chance of inheriting the mutated

locus. Screening for the targeted gene was carried out by

Southern blot analysis of StuI-digested DNA from tail

15   biopsies, using the probe utilized in identifying targeted ES

clones (probe A, Fig. 21f). As expected, approximately 50

percent of the agouti offspring showed a hybridizing StuI band

of approximately 3 kb in addition to the wild-type band of 4.7

kb, demonstrating germline transmission of the targeted J_H gene

20   segment.

In order to generate mice homozygous for the

mutation, heterozygotes were bred together and the heavy chain

genotype of the offspring determined as described above. As

expected, three genotypes were derived from the heterozygote

25   matings: wild-type mice bearing two copies of the normal J_H

locus, heterozygotes carrying one targeted copy of the gene

and one normal copy, and mice homozygous for the J_H mutation.

The absence of J_H sequences from these latter mice was verified

by hybridization of the Southern blots of StuI-digested DNA

30   with a probe specific for J_H (probe C, Fig. 21a). Whereas

hybridization of the J_H probe to a 4.7 kb fragment in DNA

samples from heterozygous and wild-type siblings was observed,

no signal was present in samples from the J_H-mutant

homozygotes, attesting to the generation of a novel mouse

strain in which both copies of the heavy chain gene have been

mutated by deletion of the J_H sequences.

EXAMPLE 12

<u>Heavy Chain Minilocus Transgene</u>

A.   <u>Construction of plasmid vectors for cloning large DNA</u>

<u>sequences</u>

1.   <u>pGP1a</u>

The plasmid pBR322 was digested with EcoRI and StyI

and ligated with the following oligonucleotides:

oligo-42   5'- caa gag ccc gcc taa tga gcg ggc ttt ttt ttg cat
            act gcg gcc gct -3'

oligo-43   5'- aat tag cgg ccg cag tat gca aaa aaa agc ccg ctc
            att agg cgg gct -3'

The resulting plasmid, pGP1a, is designed for

cloning very large DNA constructs that can be excised by the

rare cutting restriction enzyme NotI. It contains a NotI

restriction site downstream (relative to the ampicillin

resistance gene, AmpR) of a strong transcription termination

signal derived from the trpA gene (Christie et al., <u>Proc.</u>

<u>Natl. Acad. Sci. USA</u> <u>78</u>:4180 (1981)). This termination signal

reduces the potential toxicity of coding sequences inserted

into the NotI site by eliminating readthrough transcription

from the AmpR gene. In addition, this plasmid is low copy

relative to the pUC plasmids because it retains the pBR322

copy number control region. The low copy number further

reduces the potential toxicity of insert sequences and reduces

the selection against large inserts due to DNA replication.

The vectors pGP1b, pGP1c, pGP1d, and pGP1f are derived from
pGP1a and contain different polylinker cloning sites. The
polylinker sequences are given below

5

pGP1a

```
      NotI
    GCGGCCGC
```

10

pGP1b

```
   NotI   XhoI         ClaI          BamHI         HindIII   NotI

GCggccgcctcgagatcactatcgattaattaaggatccagcagtaagcttgcGGCCGC
```

15

pGI1c

20

```
   NotI     SmaI    XhoI  SalI  HindIII   BamHI SacII NotI
GCggccgcatcccgggtctcgaggtcgacaagctttcgaggatccgcGGCCGC
```

25   pGP1d

```
   NotI    SalI HindIII ClaI BamHI XhoI    NotI
GCggccgctgtcgacaagcttatcgatggatcctcgagtgcGGCCGC
```

30

pGP1f

```
   NotI    SalI HindIII EcoRI  ClaI     KpnI  BamHI XhoI     NotI
GCggccgctgtcgacaagcttcgaattcagatcgatgtggtacctggatcctcgagtgcGGCCGC
```

35

Each of these plasmids can be used for the construction of
large transgene inserts that are excisable with NotI so that
the transgene DNA can be purified away from vector sequences
prior to microinjection.

40

2.   pGP1b

pGP1a was digested with NotI and ligated with the following oligonucleotides:

oligo-47   5'- ggc cgc aag ctt act gct gga tcc tta att aat cga
            tag tga tct cga ggc -3'

oligo-48   5'- ggc cgc ctc gag atc act atc gat taa tta agg atc
            cag cag taa gct tgc -3'

The resulting plasmid, pGP1b, contains a short polylinker region flanked by NotI sites. This facilitates the construction of large inserts that can be excised by NotI digestion.

3.   pGPe

The following oligonucleotides:

oligo-44   5'- ctc cag gat cca gat atc agt acc tga aac agg gct
            tgc -3'

oligo-45   5'- ctc gag cat gca cag gac ctg gag cac aca cag cct
            tcc -3'

were used to amplify the immunoglobulin heavy chain 3' enhancer (S. Petterson, et al., Nature 344:165-168 (1990)) from rat liver DNA by the polymerase chain reaction technique.

The amplified product was digested with BamHI and SphI and cloned into BamHI/SphI digested pNN03 (pNN03 is a pUC derived plasmid that contains a polylinker with the following restriction sites, listed in order: NotI, BamHI, NcoI, ClaI, EcoRV, XbaI, SacI, XhoI, SphI, PstI, BglII, EcoRI, SmaI, KpnI, HindIII, and NotI). The resulting plasmid, pRE3, was digested with BamHI and HindIII, and the insert containing the rat Ig heavy chain 3' enhancer cloned into BamHI/HindIII digested

159

pGP1b. The resulting plasmid, pGPe (Fig. 22 and Table 1), contains several unique restriction sites into which sequences can be cloned and subsequently excised together with the 3' enhancer by NotI digestion.

TABLE 1

B.   Construction of IgM expressing minilocus transgene, pIGM1

1.   Isolation of J-μ constant region clones and construction of pJM1

5   A human placental genomic DNA library cloned into the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories, Inc., Palo Alto, CA) was screened with the human heavy chain J region specific oligonucleotide:

10   oligo-1   5'- gga ctg tgt ccc tgt gtg atg ctt ttg atg tct ggg
                 gcc aag -3'

and the phage clone λ1.3 isolated. A 6 kb HindIII/KpnI
15   fragment from this clone, containing all six J segments as well as D segment DHQ52 and the heavy chain J-μ intronic enhancer, was isolated. The same library was screened with the human μ specific oligonucleotide:

20   oligo-2   5'- cac caa gtt gac ctg cct ggt cac aga cct gac cac
                 cta tga -3'

and the phage clone λ2.1 isolated. A 10.5 kb HindIII/XhoI
25   fragment, containing the μ switch region and all of the μ constant region exons, was isolated from this clone. These two fragments were ligated together with KpnI/XhoI digested pNNO3 to obtain the plasmid pJM1.

30   2.   pJM2

           A 4 kb XhoI fragment was isolated from phage clone λ2.1 that contains sequences immediately downstream of the sequences in pJM1, including the so called $\sum\mu$ element involved in δ-associated deleteon of the μ in certain IgD expressing
35   B-cells (Yasui et al., Eur. J. Immunol. 19:1399 (1989), which

is incorporated herein by reference). This fragment was
treated with the Klenow fragment of DNA polymerase I and
ligated to XhoI cut, Klenow treated, pJM1. The resulting
plasmid, pJM2 (Fig. 23), had lost the internal XhoI site but
5   retained the 3' XhoI site due to incomplete reaction by the
Klenow enzyme. pJM2 contains the entire human J region, the
heavy chain J-μ intronic enhancer, the μ switch region and all
of the μ constant region exons, as well as the two 0.4 kb
direct repeats, $\sigma\mu$ and $\sum\mu$, involved in δ-associated deletion
10 of the μ gene.

3.   <u>Isolation of D region clones and construction of pDH1</u>
         The following human D region specific
oligonucleotide:

15

oligo-4   5'- tgg tat tac tat ggt tcg ggg agt tat tat aac cac
           agt gtc -3'

20 was used to screen the human placenta genomic library for D
region clones. Phage clones λ4.1 and λ4.3 were isolated. A
5.5 kb XhoI fragment, that includes the D elements D_{K1}, D_{N1}, and
D_{M2} (Ichihara et al., <u>EMBO J.</u> 7:4141 (1988)), was isolated from
phage clone λ4.1. An adjacent upstream 5.2 kb XhoI fragment,
25 that includes the D elements D_{LR1}, D_{XP1}, $D_{XP'1}$, and D_{A1}, was
isolated from phage clone λ4.3. Each of these D region XhoI
fragments were cloned into the SalI site of the plasmid vector
pSP72 (Promega, Madison, WI) so as to destroy the XhoI site
linking the two sequences. The upstream fragment was then
30 excised with XhoI and SmaI, and the downstream fragment with
EcoRV and XhoI. The resulting isolated fragments were ligated
together with SalI digested pSP72 to give the plasmid pDH1.

pDH1 contains a 10.6 kb insert that includes at least 7 D segments and can be excised with XhoI (5') and EcoRV (3').

4. pCOR1

5   The plasmid pJM2 was digested with Asp718 (an isoschizomer of KpnI) and the overhang filled in with the Klenow fragment of DNA polymerase I. The resulting DNA was then digested with ClaI and the insert isolated. This insert was ligated to the XhoI/EcoRV insert of pDH1 and XhoI/ClaI

10 digested pGPe to generate pCOR1 (Fig. 24).

5. pVH251

        A 10.3 kb genomic HindIII fragment containing the two human heavy chain variable region segments V_H251 and V_H105

15 (Humphries et al., Nature 331:446 (1988), which is incorporated herein by reference) was subcloned into pSP72 to give the plasmid pVH251.

6. pIGM1

20   The plasmid pCOR1 was partially digested with XhoI and the isolated XhoI/SalI insert of pVH251 cloned into the upstream XhoI site to generate the plasmid pIGM1 (Fig. 25). pIGM1 contains 2 functional human variable region segments, at least 8 human D segments all 6 human J_H segments, the human $J-\mu$

25 enhancer, the human $\sigma\mu$ element, the human μ switch region, all of the human μ coding exons, and the human $\sum\mu$ element, together with the rat heavy chain 3' enhancer, such that all of these sequence elements can be isolated on a single fragment, away from vector sequences, by digestion with NotI

30 and microinjected into mouse embryo pronuclei to generate transgenic animals.

C. Construction of IgM and IgG expressing minilocus
transgene, pHC1

1. Isolation of γ constant region clones

       The following oligonucleotide, specific for human Ig
g constant region genes:

       oligo-29   5'- cag cag gtg cac acc caa tgc cca tga gcc cag aca
                  ctg gac -3'

10   was used to screen the human genomic library. Phage clones
       129.4 and λ29.5 were isolated. A 4 kb HindIII fragment of
       phage clone λ29.4, containing a γ switch region, was used to
       probe a human placenta genomic DNA library cloned into the
       phage vector lambda FIX™ II (Stratagene, La Jolla, CA). Phage
15   clone λSg1.13 was isolated. To determine the subclass of the
       different γ clones, dideoxy sequencing reactions were carried
       out using subclones of each of the three phage clones as
       templates and the following oligonucleotide as a primer:

20   oligo-67   5'- tga gcc cag aca ctg gac -3'

       Phage clones λ29.5 and λSγ1.13 were both determined
to be of the γ1 subclass.

25 2.   pγe1

       A 7.8 kb HindIII fragment of phage clone λ29.5,
containing the γ1 coding region was cloned into pUC18. The
resulting plasmid, pLT1, was digested with XhoI, Klenow
treated, and religated to destroy the internal XhoI site. The
30 resulting clone, pLT1xk, was digested with HindIII and the
insert isolated and cloned into pSP72 to generate the plasmid
clone pLT1xks. Digestion of pLT1xks at a polylinker XhoI site

and a human sequence derived BamHI site generates a 7.6 kb

fragment containing the γ1 constant region coding exons. This

7.6 kb XhoI/BamHI fragment was cloned together with an

adjacent downstream 4.5 kb BamHI fragment from phage clone

5   λ29.5 into XhoI/BamHI digested pGPe to generate the plasmid

clone pγe1. pγe1 contains all of the γ1 constant region

coding exons, together with 5 kb of downstream sequences,

linked to the rat heavy chain 3' enhancer.

10   3.   pγe2

A 5.3 kb HindIII fragment containing the γ1 switch

region and the first exon of the pre-switch sterile transcript

(P. Sideras et al. (1989) International Immunol. 1, 631) was

isolated from phage clone λSγ1.13 and cloned into pSP72 with

15   the polylinker XhoI site adjacent to the 5' end of the insert,

to generate the plasmid clone pSγ1s. The XhoI/SalI insert of

pSγ1s was cloned into XhoI digested pγe1 to generate the

plasmid clone pγe2 (Fig. 26). pγe2 contains all of the γ1

constant region coding exons, and the upstream switch region

20   and sterile transcript exons, together with 5 kb of downstream

sequences, linked to the rat heavy chain 3' enhancer. This

clone contains a unique XhoI site at the 5' end of the insert.

The entire insert, together with the XhoI site and the 3' rat

enhancer can be excised from vector sequences by digestion

25   with NotI.

4.   pHC1

The plasmid pIGM1 was digested with XhoI and the 43

30   kb insert isolated and cloned into XhoI digested pge2 to

generate the plasmid pHC1 (Fig. 25). pHC1 contains 2

functional human variable region segments, at least 8 human D

segments all 6 human J_H segments, the human J-μ enhancer, the human $\sigma\mu$ element, the human μ switch region, all of the human μ coding exons, the human $\sum\mu$ element, and the human γ1 constant region, including the associated switch region and sterile transcript associated exons, together with the rat heavy chain 3' enhancer, such that all of these sequence elements can be isolated on a single fragment, away from vector sequences, by digestion with NotI and microinjected into mouse embryo pronuclei to generate transgenic animals.

D.   Construction of IgM and IgG expressing minilocus transgene, pHC2

1.   Isolation of human heavy chain V region gene VH49.8

The human placental genomic DNA library lambda, FIX™ II, Stratagene, La Jolla, CA) was screened with the following human VH1 family specific oligonucleotide:

oligo-49   5'- gtt aaa gag gat ttt att cac ccc tgt gtc ctc tcc aca ggt gtc -3'

Phage clone λ49.8 was isolated and a 6.1 kb XbaI fragment containing the variable segment VH49.8 subcloned into pNN03 (such that the polylinker ClaI site is downstream of VH49.8 and the polylinker XhoI site is upstream) to generate the plasmid pVH49.8. An 800 bp region of this insert was sequenced, and VH49.8 found to have an open reading frame and intact splicing and recombination signals, thus indicating that the gene is functional (Table 2).

TABLE 2

2. pV2

A 4 kb XbaI genomic fragment containing the human
V_HIV family gene V_H4-21 (Sanz et al., EMBO J., 8:3741 (1989)),
subcloned into the plasmid pUC12, was excised with SmaI and
5 HindIII, and treated with the Klenow fragment of polymerase I.
The blunt ended fragment was then cloned into ClaI digested,
Klenow treated, pVH49.8. The resulting plasmid, pV2, contains
the human heavy chain gene VH49.8 linked upstream of VH4-21 in
the same orientation, with a unique SalI site at the 3' end of
10 the insert and a unique XhoI site at the 5' end.

3. pSγ1-5'

A 0.7 kb XbaI/HindIII fragment (representing
sequences immediately upstream of, and adjacent to, the 5.3 kb
15 γ1 switch region containing fragment in the plasmid pγe2)
together with the neighboring upstream 3.1 kb XbaI fragment
were isolated from the phage clone λSg1.13 and cloned into
HindIII/XbaI digested pUC18 vector. The resulting plasmid,
pSγ1-5', contains a 3.8 kb insert representing sequences
20 upstream of the initiation site of the sterile transcript
found in B-cells prior to switching to the γ1 isotype (P.
Sideras et al., International Immunol. 1:631 (1989)). Because
the transcript is implicated in the initiation of isotype
switching, and upstream cis-acting sequences are often
25 important for transcription regulation, these sequences are
included in transgene constructs to promote correct expression
of the sterile transcript and the associated switch
recombination.

30 4. pVGE1

The pSγ1-5' insert was excised with SmaI and HindIII, treated with Klenow enzyme, and ligated with the following oligonucleotide linker:

5   5'- ccg gtc gac cgg -3'

The ligation product was digested with SalI and ligated to SalI digested pV2. The resulting plasmid, pVP, contains 3.8 kb of γ1 switch 5' flanking sequences linked downstream of the

10 two human variable gene segments VH49.8 and VH4-21 (see Table 2). The pVP insert is isolated by partial digestion with SalI and complete digestion with XhoI, followed by purification of the 15 kb fragment on an agarose gel. The insert is then cloned into the XhoI site of pγe2 to generate the plasmid

15 clone pVGE1 (Fig. 27). pVGE1 contains two human heavy chain variable gene segments upstream of the human γ1 constant gene and associated switch region. A unique SalI site between the variable and constant regions can be used to clone in D, J, and μ gene segments. The rat heavy chain 3' enhancer is

20 linked to the 3' end of the γ1 gene and the entire insert is flanked by NotI sites.

5. <u>pHC2</u>

The plasmid clone pVGE1 is digested with SalI and

25 the XhoI insert of pIGM1 is cloned into it. The resulting clone, pHC2 (Fig. 25), contains 4 functional human variable region segments, at least 8 human D segments all 6 human J_H segments, the human J-m enhancer, the human σμ element, the human μ switch region, all of the human μ coding exons, the

30 human ∑μ element, and the human γ1 constant region, including the associated switch region and sterile transcript associated exons, together with 4 kb flanking sequences upstream of the

sterile transcript initiation site. These human sequences are
linked to the rat heavy chain 3' enhancer, such that all of
the sequence elements can be isolated on a single fragment,
away from vector sequences, by digestion with NotI and

5   microinjected into mouse embryo pronuclei to generate
transgenic animals. A unique XhoI site at the 5' end of the
insert can be used to clone in additional human variable gene
segments to further expand the recombinational diversity of
this heavy chain minilocus.

10

E.   Transgenic mice

The NotI inserts of plasmids pIGM1 and pHC1 were
isolated from vector sequences by agarose gel electrophoresis.
The purified inserts were microinjected into the pronuclei of

15 fertilized (C57BL/6 x CBA)F2 mouse embryos and transferred the
surviving embryos into pseudopregnant females as described by
Hogan et al. (B. Hogan, F. Costantini, and E. Lacy, Methods of
Manipulating the Mouse Embryo, 1986, Cold Spring Harbor
Laboratory, New York). Mice that developed from injected

20 embryos were analyzed for the presence of transgene sequences
by Southern blot analysis of tail DNA. Transgene copy number
was estimated by band intensity relative to control standards
containing known quantities of cloned DNA. At 3 to 8 weeks of
age, serum was isolated from these animals and assayed for the

25 presence of transgene encoded human IgM and IgG1 by ELISA as
described by Harlow and Lane (E. Harlow and D. Lane.
Antibodies: A Laboratory Manual, 1988, Cold Spring Harbor
Laboratory, New York). Microtiter plate wells were coated
with mouse monoclonal antibodies specific for human IgM (clone

30 AF6, #0285, AMAC, Inc. Westbrook, ME) and human IgG1 (clone
JL512, #0280, AMAC, Inc. Westbrook, ME). Serum samples were
serially diluted into the wells and the presence of specific

immunoglobulins detected with affinity isolated alkaline

phosphatase conjugated goat anti-human Ig (polyvalent) that

had been pre-adsorbed to minimize cross-reactivity with mouse

immunoglobulins. Table 3 and Fig. 28 show the results of an

5   ELISA assay for the presence of human IgM and IgG1 in the

serum of two animals that developed from embryos injected with

the transgene insert of plasmid pHC1. All of the control non-

transgenic mice tested negative for expression of human IgM

and IgG1 by this assay. Mice from two lines containing the

10   pIGM1 NotI insert (lines #6 and 15) express human IgM but not

human IgG1. We tested mice from 6 lines that contain the pHC1

insert and found that 4 of the lines (lines #26, 38, 57 and

122) express both human IgM and human IgG1, while mice from

two of the lines (lines #19 and 21) do not express detectable

15   levels of human immunoglobulins. The pHC1 transgenic mice

that did not express human immunoglobulins were so-called G_o

mice that developed directly from microinjected embryos and

may have been mosaic for the presence of the transgene.

Southern blot analysis indicates that many of these mice

20   contain one or fewer copies of the transgene per cell. The

detection of human IgM in the serum of pIGM1 transgenics, and

human IgM and IgG1 in pHC1 transgenics, provides evidence that

the transgene sequences function correctly in directing VDJ

joining, transcription, and isotype switching. One of the

25   animals (#18) was negative for the transgene by Southern blot

analysis, and showed no detectable levels of human IgM or

IgG1. The second animal (#38) contained approximately 5

copies of the transgene, as assayed by Southern blotting, and

showed detectable levels of both human IgM and IgG1. The

30   results of ELISA assays for 11 animals that developed from

transgene injected embryos is summarized in the table below

(Table 3).

TABLE 3

Detection of human IgM and IgG1 in the serum of transgenic animals by ELISA assay

animal #	injected transgene	approximate transgene copies per cell	human IgM	human IgG1
6	pIGM1	1	++	-
7	pIGM1	0	-	-
9	pIGM1	0	-	-
10	pIGM1	0	-	-
12	pIGM1	0	-	-
15	pIGM1	10	++	-
18	pHC1	0	-	-
19	pHC1	1	-	-
21	pHC1	<1	-	-
26	pHC1	2	++	+
38	pHC1	5	++	+

Table 3 shows a correlation between the presence of integrated transgene DNA and the presence of transgene encoded immunoglobulins in the serum. Two of the animals that were found to contain the pHC1 transgene did not express detectable levels of human immunoglobulins. These were both low copy animals and may not have contained complete copies of the transgenes, or the animals may have been genetic mosaics

(indicated by the <1 copy per cell estimated for animal #21),
and the transgene containing cells may not have populated the
hematopoietic lineage. Alternatively, the transgenes may have
integrated into genomic locations that are not conducive to
5   their expression. The detection of human IgM in the serum of
pIGM1 transgenics, and human IgM and IgG1 in pHC1 transgenics,
indicates that the transgene sequences function correctly in
directing VDJ joining, transcription, and isotype switching.

10   F.   cDNA clones

          To assess the functionality of the pHC1 transgene in
VDJ joining and class switching, as well the participation of
the transgene encoded human B-cell receptor in B-cell
development and allelic exclusion, the structure of
15   immunoglobulin cDNA clones derived from transgenic mouse
spleen mRNA were examined. The overall diversity of the
transgene encoded heavy chains, focusing on D and J segment
usage, N region addition, CDR3 length distribution, and the
frequency of joints resulting in functional mRNA molecules was
20   examined. Transcripts encoding IgM and IgG incorporating
VH105 and VH251 were examined.

          Polyadenylated RNA was isolated from an eleven week
old male second generation line-57 pHC1 transgenic mouse.
This RNA was used to synthesize oligo-dT primed single
25   stranded cDNA. The resulting cDNA was then used as template
for four individual PCR amplifications using the following
four synthetic oligonucleotides as primers: VH251 specific
oligo-149, cta gct cga gtc caa gga gtc tgt gcc gag gtg cag ctg
(g,a,t,c); VH105 specific o-150, gtt gct cga gtg aaa ggt gtc
30   cag tgt gag gtg cag ctg (g,a,t,c); human gamma1 specific
oligo-151, ggc gct cga gtt cca cga cac cgt cac cgg ttc; and
human mu specific oligo-152, cct gct cga ggc agc caa cgg cca

cgc tgc tcg. Reaction 1 used primers o-149 and o-151 to
amplify VH251-gammal transcripts, reaction 2 used o-149 and o-
152 to amplify VH251-mu transcripts, reaction 3 used o-150 and
o-151 to amplify VH105-gammal transcripts, and reaction 4 used
5   o-150 and o-152 to amplify VH105-mu transcripts. The
resulting 0.5 kb PCR products were isolated from an agarose
gel; the μ transcript products were more abundant than the γ
transcript products, consistent with the corresponding ELISA
data (Fig. 34). The PCR products were digested with XhoI and
10   cloned into the plasmid pNN03. Double-stranded plasmid DNA
was isolated from minipreps of nine clones from each of the
four PCR amplifications and dideoxy sequencing reactions were
performed. Two of the clones turned out to be deletions
containing no D or J segments. These could not have been
15   derived from normal RNA splicing products and are likely to
have originated from deletions introduced during PCR
amplification. One of the DNA samples turned out to be a
mixture of two individual clones, and three additional clones
did not produce readable DNA sequence (presumably because the
20   DNA samples were not clean enough). The DNA sequences of the
VDJ joints from the remaining 30 clones are compiled in Table
4. Each of the sequences are unique, indicating that no
single pathway of gene rearrangement, or single clone of
transgene expressing B-cells is dominant. The fact that no
25   two sequences are alike is also an indication of the large
diversity of immunoglobulins that can be expressed from a
compact minilocus containing only 2 V segments, 10 D segments,
and 6 J segments. Both of the V segments, all six of the J
segments, and 7 of the 10 D segments that are included in the
30   transgene are used in VDJ joints. In addition, both constant
region genes (mu and gammal) are incorporated into
transcripts. The VH105 primer turned out not to be specific

for VH105 in the reactions performed. Therefore many of the clones from reactions 3 and 4 contained VH251 transcripts. Additionally, clones isolated from ligated reaction 3 PCR product turned out to encode IgM rather than IgG; however this

5. may reflect contamination with PCR product from reaction 4 as the DNA was isolated on the same gel. An analogous experiment, in which immunoglobulin heavy chain sequences were amplified from adult human peripheral blood lymphocytes (PBL), and the DNA sequence of the VDJ joints determined, was

10 recently reported by Yamada et al. (J. Exp. Med. 173:395-407 (1991), which is incorporated herein by reference). We compared the data from human PBL with our data from the pHC1 transgenic mouse.

TABLE 4

G.   <u>J segment choice</u>

        Table 5 compared the distribution of J segments
incorporated into pHC1 transgene encoded transcripts to J
segments found in adult human PBL immunoglobulin transcripts.
5   The distribution profiles are very similar, J4 is the dominant
segment in both systems, followed by J6. J2 is the least
common segment in human PBL and the transgenic animal.

<u>TABLE 5</u>   J. Segment Choice
10

                                      Percent Usage (± 3%)
              <u>J. Segment</u>   <u>HC1 transgenic</u>   <u>Human PBL</u>

                J1   7   1
15   J2   3   <1
                J3   17   9
                J4   44   53
                J5   3   15
                J6   <u> 26 </u>   <u> 22 </u>
20
                                   100%   100%

H.   <u>D segment choice</u>

25   49% (40 of 82) of the clones analyzed by Yamada et
al. incorporated D segments that are included in the pHC1
transgene.   An additional 11 clones contained sequences that
were not assigned by the authors to any of the known D
segments.   Two of these 11 unassigned clones appear to be
30   derived from an inversion of the DIR2 segments which is
included in the pHC1 construct. This mechanism, which was
predicted by Ichihara et al. (<u>EMBO J</u>. <u>7</u>:4141 (1988)) and
observed by Sanz (<u>J. Immunol</u>. <u>147</u>:1720-1729 (1991)), was not
considered by Yamada et al. (<u>J. Exp. Med</u>. <u>173</u>:395-407 (1991)).
35   Table 5 is a comparison of the D segment distribution for the
pHC1 transgenic mouse and that observed for human PBL
transcripts by Yamada et al. The data of Yamada et al. was

recompiled to include DIR2 use, and to exclude D segments that
are not in the pHC1 transgene. Table 6 demonstrates that the
distribution of D segment incorporation is very similar in the
transgenic mouse and in human PBL. The two dominant human D

5   segments, DXP'1 and DN1, are also found with high frequency in
the transgenic mouse. The most dramatic dissimilarity between
the two distributions is the high frequency of DHQ52 in the
transgenic mouse as compared to the human. The high frequency
of DHQ52 is reminiscent of the D segment distribution in the

10   human fetal liver. Sanz has observed that 14% of the heavy
chain transcripts contained DHQ52 sequences. If D segments
not found in pHC1 are excluded from the analysis, 31% of the
fetal transcripts analyzed by Sanz contain DHQ52. This is
comparable to the 27% that we observe in the pHC1 transgenic

15   mouse.

TABLE 6   D Segment Choice

		Percent Usage (± 3%)	
	D. Segment	HC1 transgenic	Human PBL
	DLR1	<1	<1
	DXP1	3	6
	DXP'1	25	19
	DA1	<1	12
	DK1	7	12
	DN1	12	22
	DIR2	7	4
	DM2	<1	2
	DLR2	3	4
	DHQ52	26	2
	?	17	17
		100%	100%

I.   Functionality of VDJ joints

     Table 7 shows the predicted amino acid sequences of
the VDJ regions from 30 clones that were analyzed from the

pHC1 transgenic. The translated sequences indicate that 23 of the 30 VDJ joints (77%) are in-frame with respect to the variable and J segments.

5

<u>TABLE 7</u>   Functionality of V-D-J Joints

J.   CDR3 length distribution

        Table 8 compared the length of the CDR3 peptides
from transcripts with in-frame VDJ joints in the pHC1
transgenic mouse to those in human PBL. Again the human PBL
5 data comes from Yamada et al. The profiles are similar with
the transgenic profile skewed slightly toward smaller CDR3
peptides than observed from human PBL. The average length of
CDR3 in the transgenic mouse is 10.3 amino acids. This is
substantially the same as the average size reported for
10 authentic human CDR3 peptides by Sanz (J. Immunol. 147:1720-
1729 (1991)).

    TABLE 8   CDR3 Length Distribution

15

                                   Percent Occurrence (± 3%)
            #amino acids in CDR3   HC1 transgenic   Human PBL

                3-8   26   14
20   9-12   48   41
                13-18   26   37
                19-23   <1   7
                >23   <1   1

25   100%   100%

                        EXAMPLE 13

30 Rearranged Heavy Chain Transgenes

    A.   Isolation of Rearranged Human Heavy Chain VDJ segments.
        Two human leukocyte genomic DNA libraries cloned
into the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories,
Inc., Palo Alto, CA) are screened with a 1 kb PacI/HindIII
35 fragment of λ1.3 containing the human heavy chain J-μ intronic
enhancer. Positive clones are tested for hybridization with a
mixture of the following V$_H$ specific oligonucleotides:

oligo-7   5'-tca gtg aag gtt tcc tgc aag gca tct gga tac acc

           ttc acc-3'

oligo-8   5'-tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc

5   ttc agt-3'

           Clones that hybridized with both V and J-μ probes

are isolated and the DNA sequence of the rearranged VDJ

segment determined.

10

   B.   Construction of rearranged human heavy chain transgenes

           Fragments containing functional VJ segments (open

reading frame and splice signals) are subcloned into the

plasmid vector pSP72 such that the plasmid derived XhoI site

15 is adjacent to the 5' end of the insert sequence. A subclone

containing a functional VDJ segment is digested with XhoI and

PacI (PacI, a rare-cutting enzyme, recognizes a site near the

J-m intronic enhancer), and the insert cloned into XhoI/PacI

digested pHC2 to generate a transgene construct with a

20 functional VDJ segment, the J-μ intronic enhancer, the μ

switch element, the μ constant region coding exons, and the γ1

constant region, including the sterile transcript associated

sequences, the γ1 switch, and the coding exons. This

transgene construct is excised with NotI and microinjected

25 into the pronuclei of mouse embryos to generate transgenic

animals as described above.

                              EXAMPLE 14

Light Chain Transgenes

30 A.   Construction of Plasmid vectors

   1.   Plasmid vector pGP1c

Plasmid vector pGP1a is digested with NotI and the following oligonucleotides ligated in:

oligo-81   5'-ggc cgc atc ccg ggt ctc gag gtc gac aag ctt tcg
5   agg atc cgc-3'

oligo-82   5'-ggc cgc gga tcc tcg aaa gct tgt cga cct cga gac
            ccg gga tgc-3'

10 The resulting plasmid, pGP1c, contains a polylinker with XmaI,
    XhoI, SalI, HindIII, and BamHI restriction sites flanked by
    NotI sites.

    2. Plasmid vector pGP1d
15   Plasmid vector pGP1a is digested with NotI and the
    following oligonucleotides ligated in:

    oligo-87   5'-ggc cgc tgt cga caa gct tat cga tgg atc ctc gag
                tgc -3'
20

    oligo-88   5'-ggc cgc act cga gga tcc atc gat aag ctt gtc gac
                agc -3'

    The resulting plasmid, pGP1d, contains a polylinker with SalI,
25 HindIII, ClaI, BamHI, and XhoI restriction sites flanked by
    NotI sites.

    B. <u>Isolation of Jκ and Cκ clones</u>
            A human placental genomic DNA library cloned into
30 the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories, Inc.,
    Palo Alto, CA) was screened with the human kappa light chain J
    region specific oligonucleotide:

oligo-36 5'- cac ctt cgg cca agg gac acg act gga gat taa acg
           taa gca -3'

and the phage clones 136.2 and 136.5 isolated. A 7.4 kb XhoI
5 fragment that includes the JK1 segment was isolated from
   136.2 and subcloned into the plasmid pNNO3 to generate the
   plasmid clone p36.2. A neighboring 13 kb XhoI fragment that
   includes Jk segments 2 through 5 together with the CK gene
   segment was isolated from phage clone 136.5 and subcloned into
10 the plasmid pNNO3 to generate the plasmid clone p36.5.
   Together these two clones span the region beginning 7.2 kb
   upstream of JK1 and ending 9 kb downstream of CK.

C.   Construction of rearranged light chain transgenes
15 1. pCK1, a CK vector for expressing rearranged variable
   segments
          The 13 kb XhoI insert of plasmid clone p36.5
   containing the CK gene, together with 9 kb of downstream
   sequences, is cloned into the SalI site of plasmid vector
20 pGP1c with the 5' end of the insert adjacent to the plasmid
   XhoI site. The resulting clone, pCK1 can accept cloned
   fragments containing rearranged VJK segments into the unique
   5' XhoI site. The transgene can then be excised with NotI and
   purified from vector sequences by gel electrophoresis. The
25 resulting transgene construct will contain the human J-CK
   intronic enhancer and may contain the human 3' κ enhancer.

2. pCK2, a CK vector with heavy chain enhancers for
   expressing rearranged variable segments
30   A 0.9 kb XbaI fragment of mouse genomic DNA
   containing the mouse heavy chain J-μ intronic enhancer (J.
   Banerji et al., Cell 33:729-740 (1983)) was subcloned into

pUC18 to generate the plasmid pJH22.1. This plasmid was
linearized with SphI and the ends filled in with Klenow
enzyme. The Klenow treated DNA was then digested with HindIII
and a 1.4 kb MluI/HindIII fragment of phage clone λ1.3

5   (previous example), containing the human heavy chain J-μ
intronic enhancer (Hayday et al., Nature 307:334-340 (1984)),
to it. The resulting plasmid, pMHE1, consists of the mouse
and human heavy chain J-μ intronic enhancers ligated together
into pUC18 such that they are excised on a single

10   BamHI/HindIII fragment. This 2.3 kb fragment is isolated and
cloned into pGP1c to generate pMHE2. pMHE2 is digested with
SalI and the 13 kb XhoI insert of p36.5 cloned in. The
resulting plasmid, pCK2, is identical to pCK1, except that the
mouse and human heavy chain J-μ intronic enhancers are fused

15   to the 3' end of the transgene insert. To modulate expression
of the final transgene, analogous constructs can be generated
with different enhancers, i.e. the mouse or rat 3' kappa or
heavy chain enhancer (Meyer and Neuberger, EMBO J.,
8:1959-1964 (1989); Petterson et al., Nature, 344:165-168

20   (1990)).

3.   Isolation of rearranged kappa light chain variable
segments
             Two human leukocyte genomic DNA libraries cloned

25   into the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories,
Inc., Palo Alto, CA) were screened with the human kappa light
chain J region containing 3.5 kb XhoI/SmaI fragment of p36.5.
Positive clones were tested for hybridization with the
following Vκ specific oligonucleotide:

30

oligo-65   5'-agg ttc agt ggc agt ggg tct ggg aca gac ttc act
            ctc acc atc agc-3'

Clones that hybridized with both V and J probes are isolated
and the DNA sequence of the rearranged VJκ segment determined.

4.   Generation of transgenic mice containing rearranged human
5   light chain constructs.

        Fragments containing functional VJ segments (open
reading frame and splice signals) are subcloned into the
unique XhoI sites of vectors pCK1 and pCK2 to generate
rearranged kappa light chain transgenes. The transgene
10 constructs are isolated from vector sequences by digestion
with NotI. Agarose gel purified insert is microinjected into
mouse embryo pronuclei to generate transgenic animals.
Animals expressing human kappa chain are bred with heavy chain
minilocus containing transgenic animals to generate mice
15 expressing fully human antibodies.

        Because not all VJκ combinations may be capable of
forming stable heavy-light chain complexes with a broad
spectrum of different heavy chain VDJ combinations, several
different light chain transgene constructs are generated, each
20 using a different rearranged VJk clone, and transgenic mice
that result from these constructs are bred with heavy chain
minilocus transgene expressing mice. Peripheral blood,
spleen, and lymph node lymphocytes are isolated from double
transgenic (both heavy and light chain constructs) animals,
25 stained with fluorescent antibodies specific for human and
mouse heavy and light chain immunoglobulins (Pharmingen, San
Diego, CA) and analyzed by flow cytometry using a FACScan
analyzer (Becton Dickinson, San Jose, CA). Rearranged light
chain transgenes constructs that result in the highest level
30 of human heavy/light chain complexes on the surface of the
highest number of B cells, and do not adversely affect the
immune cell compartment (as assayed by flow cytometric

analysis with B and T cell subset specific antibodies), are selected for the generation of human monoclonal antibodies.

D.   Construction of unrearranged light chain minilocus
5   transgenes

    1.   pJCK1, a Jκ, Cκ containing vector for constructing minilocus transgenes

         The 13 kb Cκ containing XhoI insert of p36.5 is treated with Klenow enzyme and cloned into HindIII digested,
10   Klenow-treated, plasmid pGP1d. A plasmid clone is selected such that the 5' end of the insert is adjacent to the vector derived ClaI site. The resulting plasmid, p36.5-1d, is digested with ClaI and Klenow-treated. The Jκ1 containing 7.4 kb XhoI insert of p36.2 is then Klenow-treated and cloned into
15   the ClaI, Klenow-treated p36.5-1d. A clone is selected in which the p36.2 insert is in the same orientation as the p36.5 insert. This clone, pJCK1 (Fig. 34), contains the entire human Jκ region and Cκ, together with 7.2 kb of upstream sequences and 9 kb of downstream sequences. The insert also
20   contains the human J-Cκ intronic enhancer and may contain a human 3' κ enhancer. The insert is flanked by a unique 3' SalI site for the purpose of cloning additional 3' flanking sequences such as heavy chain or light chain enhancers. A unique XhoI site is located at the 5' end of the insert for
25   the purpose of cloning in unrearranged Vκ gene segments. The unique SalI and XhoI sites are in turn flanked by NotI sites that are used to isolate the completed transgene construct away from vector sequences.

30   2.   Isolation of unrearranged Vκ gene segments and generation of transgenic animals expressing human Ig light chain protein

The Vκ specific oligonucleotide, oligo-65 (discussed above), is used to probe a human placental genomic DNA library cloned into the phage vector lEMBL3/SP6/T7 (Clonetech Laboratories, Inc., Palo Alto, CA). Variable gene segments

5   from the resulting clones are sequenced, and clones that appear functional are selected. Criteria for judging functionality include: open reading frames, intact splice acceptor and donor sequences, and intact recombination sequence. DNA fragments containing selected variable gene

10   segments are cloned into the unique XhoI site of plasmid pJCK1 to generate minilocus constructs. The resulting clones are digested with NotI and the inserts isolated and injected into mouse embryo pronuclei to generate transgenic animals. The transgenes of these animals will undergo V to J joining in

15   developing B-cells. Animals expressing human kappa chain are bred with heavy chain minilocus containing transgenic animals to generate mice expressing fully human antibodies.

EXAMPLE 15

20   Genomic Heavy Chain Human Ig Transgene

This Example describes the cloning of a human genomic heavy chain immunoglobulin transgene which is then introduced into the murine germline via microinjection into zygotes or integration in ES cells.

25   Nuclei are isolated from fresh human placental tissue as described by Marzluff, W.F., et al. (1985), Transcription and Translation: A Practical Approach, B.D. Hammes and S.J. Higgins, eds., pp. 89-129, IRL Press, Oxford). The isolated nuclei (or PBS washed human spermatocytes) are

30   embedded in 0.5% low melting point agarose blocks and lysed with 1 mg/ml proteinase K in 500mM EDTA, 1% SDS for nuclei, or with 1mg/ml proteinase K in 500mM EDTA, 1% SDS, 10mM DTT for

spermatocytes at 50°C for 18 hours. The proteinase K is
inactivated by incubating the blocks in 40μg/ml PMSF in TE for
30 minutes at 50°C, and then washing extensively with TE. The
DNA is then digested in the agarose with the restriction

5 enzyme NotI as described by M. Finney in Current Protocols in
Molecular Biology (F. Ausubel et al., eds. John Wiley & Sons,
Supp. 4, 1988, e.g., Section 2.5.1).

The NotI digested DNA is then fractionated by pulsed
field gel electrophoresis as described by Anand et al., Nuc.

10 Acids Res. 17:3425-3433 (1989). Fractions enriched for the
NotI fragment are assayed by Southern hybridization to detect
one or more of the sequences encoded by this fragment. Such
sequences include the heavy chain D segments, J segments, and
γ1 constant regions together with representatives of all 6 V_H

15 families (although this fragment is identified as 670 kb
fragment from HeLa cells by Berman et al. (1988), supra., we
have found it to be an 830 kb fragment from human placental
and sperm DNA). Those fractions containing this NotI
fragment are ligated into the NotI cloning site of the vector

20 pYACNN as described (McCormick et al., Technique 2:65-71
(1990)). Plasmid pYACNN is prepared by digestion of pYACneo
(Clontech) with EcoRI and ligation in the presence of the
oligonucleotide 5' - AAT TGC GGC CGC - 3'.

YAC clones containing the heavy chain NotI fragment

25 are isolated as described by Traver et al., Proc. Natl. Acad.
Sci. USA, 86:5898-5902 (1989). The cloned NotI insert is
isolated from high molecular weight yeast DNA by pulse field
gel electrophoresis as described by M. Finney, op. cit. The
DNA is condensed by the addition of 1 mM spermine and

30 microinjected directly into the nucleus of single cell embryos
previously described. Alternatively, the DNA is isolated by
pulsed field gel electrophoresis and introduced into ES cells

by lipofection (Gnirke et al., EMBO J. 10:1629-1634 (1991)),
or the YAC is introduced into ES cells by spheroplast fusion.

                        EXAMPLE 16

5   Discontinuous Genomic Heavy Chain Ig Transgene

            An 85 kb SpeI fragment of human genomic DNA,
    containing V_H6, D segments, J segments, the μ constant region
    and part of the γ constant region, has been isolated by YAC
    cloning essentially as described in Example 1. A YAC carrying
10 a fragment from the germline variable region, such as a 570 kb
    NotI fragment upstream of the 670-830 kb NotI fragment
    described above containing multiple copies of V_1 through V_5, is
    isolated as described. (Berman et al. (1988), supra. detected
    two 570 kb NotI fragments, each containing multiple V
15 segments.) The two fragments are coinjected into the nucleus
    of a mouse single cell embryo as described in Example 1.

            Typically, coinjection of two different DNA
    fragments result in the integration of both fragments at the
    same insertion site within the chromosome.   Therefore,
20 approximately 50% of the resulting transgenic animals that
    contain at least one copy of each of the two fragments will
    have the V segment fragment inserted upstream of the constant
    region containing fragment. Of these animals, about 50% will
    carry out V to DJ joining by DNA inversion and about 50% by
25 deletion, depending on the orientation of the 570 kb NotI
    fragment relative to the position of the 85 kb SpeI fragment.
    DNA is isolated from resultant transgenic animals and those
    animals found to be containing both transgenes by Southern
    blot hybridization (specifically, those animals containing
30 both multiple human V segments and human constant region
    genes) are tested for their ability to express human

immunoglobulin molecules in accordance with standard
techniques.

                          EXAMPLE 17

5 <u>Identification of functionally rearranged variable region
   sequences in transgenic B cells</u>

          An antigen of interest is used to immunize (see
   Harlow and Lane, <u>Antibodies: A Laboratory Manual</u>, Cold Spring
   Harbor, New York (1988)) a mouse with the following genetic
10 traits: homozygosity at the endogenous having chain locus for
   a deletion of J_H (Examples 10); hemizygous for a single copy of
   unrearranged human heavy chain minilocus transgene (examples 5
   and 14); and hemizygous for a single copy of a rearranged
   human kappa light chain transgene (Examples 6 and 14).
15   Following the schedule of immunization, the spleen
   is removed, and spleen cells used to generate hybridomas.
   Cells from an individual hybridoma clone that secretes
   antibodies reactive with the antigen of interest are used to
   prepare genomic DNA. A sample of the genomic DNA is digested
20 with several different restriction enzymes that recognize
   unique six base pair sequences, and fractionated on an agarose
   gel. Southern blot hybridization is used to identify two DNA
   fragments in the 2-10 kb range, one of which contains the
   single copy of the rearranged human heavy chain VDJ sequences
25 and one of which contains the single copy of the rearranged
   human light chain VJ sequence. These two fragments are size
   fractionated on agarose gel and cloned directly into pUC18.
   The cloned inserts are then subcloned respectively into heavy
   and light chain expression cassettes that contain constant
30 region sequences.
          The plasmid clone pγe1 (Example 12) is used as a
   heavy chain expression cassette and rearranged VDJ sequences

are cloned into the XhoI site. The plasmid clone pCK1 is used as a light chain expression cassette and rearranged VJ sequences are cloned into the XhoI site. The resulting clones are used together to transfect SP_0 cells to produce antibodies that react with the antigen of interest (Co. et al., Proc. Natl. Acad. Sci. USA 88:2869 (1991), which is incorporated herein by reference).

Alternatively, mRNA is isolated from the cloned hybridoma cells described above, and used to synthesize cDNA. The expressed human heavy and light chain VDJ and VJ sequence are then amplified by PCR and cloned (Larrick et al., Biol. Technology, 7:934-938 (1989)). After the nucleotide sequence of these clones has been determined, oligonucleotides are synthesized that encode the same polypeptides, and synthetic expression vectors generated as described by Queen et al., Proc. Natl. Acad. Sci. USA., 84:5454-5458 (1989).

Immunization of Transgenic Animals with Complex Antigens

The following experiment demonstrates that transgenic animals can be successfully immunized with complex antigens such as those on human red blood cells and respond with kinetics that are similar to the response kinetics observed in normal mice.

Blood cells generally are suitable immunogens and comprise many different types of antigens on the surface of red and white blood cells.

Immunization with human blood

Tubes of human blood from a single donor were collected and used to immunize transgenic mice having functionally disrupted endogenous heavy chain loci (J_HD) and harboring a human heavy chain minigene construct (HC1); these

mice are designated as line 112. Blood was washed and
resuspended in 50 mls Hanks' and diluted to 1x10^8 cells/ml 0.2
mls (2x10^7 cells) were then injected interperitoneally using a
28 gauge needle and 1 cc syringe. This immunization protocol

5   was repeated approximately weekly for 6 weeks. Serum titers
were monitored by taking blood from retro-orbital bleeds and
collecting serum and later testing for specific antibody. A
pre-immune bleed was also taken as a control. On the very
last immunization, three days before these animals were

10   sacrificed for serum and for hybridomas, a single immunization
of 1 x 10^7 cells was given intravenously through the tail to
enhance the production of hybridomas.

Table 9

15   Animals

	Mouse ID	Line	Sex	HC1-112	JHD
1	2343	112	M	+	++
2	2344	112	M	-	+
3	2345	112	F	-	+
4	2346	112	F	-	++
5	2347	112	F	-	++
6	2348	112	F	+	++
7	2349	112	F	-	+

25

Mice # 2343 and 2348 have a desired phenotype: human heavy
chain mini-gene transgenic on heavy chain knock-out
background.

30

Generation of Hybridomas

            Hybridomas were generated by fusing mouse spleen
cells of approximately 16 week-old transgenic mice (Table 9)
that had been immunized as described (supra) to a fusion
5   partner consisting of the non-secreting HAT-sensitive myeloma
cell line, X63 Ag8.653. Hybridoma clones were cultivated and
hybridoma supernatants containing immunoglobulins having
specific binding affinity for blood cell antigens were
identified, for example, by flow cytometry.
10

Flow cytometry

            Serum and hybridoma supernatants were tested using
flow cytometry. Red blood cells from the donor were washed 4X
in Hanks' balanced salt solution and 50,000 cells were placed
15 in 1.1 ml polypropylene microtubes. Cells were incubated with
antisera or supernatant from the hybridomas for 30 minutes on
ice in staining media (1x RPMI 1640 media without phenol red
or biotin (Irvine Scientific) 3% newborn calf serum, 0.1% Na
azide). Controls consisted of littermate mice with other
20 genotypes. Cells were then washed by centrifugation at 4°C in
Sorvall RT600B for 5-10 minutes at 1000 rpm. Cells were
washed two times and then antibody detected on the cell
surface with a fluorescent developing reagent. Two monoclonal
reagents were used to test. One was a FITC-labeled mouse
25 anti-human μ heavy chain antibody (Pharmagen, San Diego, CA)
and the other was a PE-labeled rat anti-mouse kappa light
chain (Becton-Dickenson, San Jose, CA). Both of these
reagents gave similar results. Whole blood (red blood cells
and white blood cells) and white blood cells alone were used
30 as target cells. Both sets gave positive results.
            Serum of transgenic mice and littermate controls was
incubated with either red blood cells from the donor, or white

blood cells from another individual, washed and then developed
with anti-human IgM FITC labeled antibody and analyzed in a
flow cytometer. Results showed that serum from mice that are
transgenic for the human mini-gene locus (mice 2343 and 2348)

5   show human IgM reactivity whereas all littermate animals
(2344, 2345, 2346, 2347) do not. Normal mouse serum (NS) and
phosphate buffer saline (PBS) were used as negative controls.
Red blood cells were ungated and white blood cells were gated
to include only lymphocytes. Lines are drawn on the x and y

10 axis to provide a reference. Flow cytometry was performed on
100 supernatants from fusion 2348. Four supernatants showed
positive reactivity for blood cell antigens.

EXAMPLE 18

15 Reduction of Endogenous Mouse Immunoglobulin Expression
by Antisense RNA

        A.   Vector for Expression of Antisense Ig Sequences
              1.   Construction of the cloning vector pGP1h
              The vector pGP1b (referred to in a previous example)

20 is digested with XhoI and BamHI and ligated with the following
oligonucleotides:

5'- gat cct cga gac cag gta cca gat ctt gtg aat tcg -3'
5'- tcg acg aat tca caa gat ctg gta cct ggt ctc gag -3'

25

to generate the plasmid pGP1h. This plasmid contains a
polylinker that includes the following restriction sites:
NotI, EcoRI, BglII, Asp718, XhoI, BamHI, HindIII, NotI.

30   Construction of pBCE1.

              A 0.8 kb XbaI/BglII fragment of pVH251 (referred to
in a previous example), that includes the promoter leader

sequence exon, first intron, and part of the second exon of the human VH-V family immunoglobulin variable gene segment, was inserted into XbaI/Bg1II digested vector pNN03 to generate the plasmid pVH251.

5   The 2.2 kb BamHI/EcoRI DNA fragment that includes the coding exons of the human growth hormone gene (hGH; Seeburg, (1982) DNA 1:239-249) is cloned into Bg1II/EcoRI digested pGH1h. The resulting plasmid is digested with BamHI and the BamHI/Bg1II of pVH251N is inserted in the same

10   orientation as the hGH gene to generate the plasmid pVhgh.

A 0.9 kb XbaI fragment of mouse genomic DNA containing the mouse heavy chain J-μ intronic enhancer (Banerji et al., (1983) Cell 33:729-740) was subcloned into pUC18 to generate the plasmid pJH22.1. This plasmid was

15   linearized with SphI and the ends filled in with klenow enzyme. The klenow treated DNA was then digested with HindIII and a 1.4 kb MluI(klenow)/HindIII fragment of phage clone λ1.3 (previous example), containing the human heavy chain J-μ intronic enhancer (Hayday et al., (1984) Nature 307:334-340),

20   to it. The resulting plasmid, pMHE1, consists of the mouse and human heavy chain J-μ intron enhancers ligated together into pUC18 such that they can be excised on a single BamHI/HindIII fragment.

The BamHI/HindIII fragment of pMHE1 is cloned into

25   BamHI/HindIII cut pVhgh to generate the B-cell expression vector pBCE1. This vector, depicted in Fig. 36, contains unique XhoI and Asp718 cloning sites into which antisense DNA fragments can be cloned. The expression of these antisense sequences is driven by the upstream heavy chain promoter-

30   enhancer combination the downstream hGH gene sequences provide polyadenylation sequences in addition to intron sequences that promote the expression of transgene constructs. Antisense

transgene constructs generated from pBCE1 can be separated from vector sequences by digestion with NotI.

   B.   An IgM antisense transgene construct.

5   The following two oligonucleotides:

      5'- cgc ggt acc gag agt cag tcc ttc cca aat gtc -3'
      5'- cgc ctc gag aca gct gga atg ggc aca tgc aga -3'

10   are used as primers for the amplification of mouse IgM constant region sequences by polymerase chain reaction (PCR) using mouse spleen cDNA as a substrate. The resulting 0.3 kb PCR product is digested with Asp718 and XhoI and cloned into Asp718/XhoI digested pBCE1 to generate the antisense transgene

15   construct pMAS1. The purified NotI insert of pMAS1 is microinjected into the pronuclei of half day mouse embryos-- alone or in combination with one or more other transgene constructs--to generate transgenic mice. This construct expresses an RNA transcript in B-cells that hybridizes with

20   mouse IgM mRNA, thus down-regulating the expression of mouse IgM protein. Double transgenic mice containing pMAS1 and a human heavy chain transgene minilocus such as pHC1 (generated either by coinjection of both constructs or by breeding of singly transgenic mice) will express the human transgene

25   encoded Ig receptor on a higher percentage of B-cell than mice transgenic for the human heavy chain minilocus alone. The ratio of human to mouse Ig receptor expressing cells is due in part to competition between the two populations for factors and cells that promoter B-cell differentiation and expansion.

30   Because the Ig receptor plays a key role in B-cell development, mouse Ig receptor expressing B-cells that express reduced levels of IgM on their surface (due to mouse Ig

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specific antisense down-regulation) during B-cell development
will not compete as well as cells that express the human
receptor.

5   C.   An IgKappa antisense transgene construct.
              The following two oligonucleotides:

    5'- cgc ggt acc gct gat gct gca cca act gta tcc -3'
    5'- cgc ctc gag cta aca ctc att cct gtt gaa gct -3'
10

    are used as primers for the amplification of mouse IgKappa
    constant region sequences by polymerase chain reaction (PCR)
    using mouse spleen cDNA as a substrate. The resulting 0.3 kb
    PCR product is digested with Asp718 and XhoI and cloned into
15 Asp718/XhoI digested pBCE1 to generate the antisense transgene
    construct pKAS1. The purified NotI insert of pKAS1 is
    microinjected into the pronuclei of half day mouse embryos--
    alone or in combination with one or more other transgene
    constructs--to generate transgenic mice. This construct
20 expresses an RNA transcript in B-cells that hybridizes with
    mouse IgK mRNA, thus down-regulating the expression of mouse
    IgK protein as described above for pMAS1.

                        EXAMPLE 19
25   This example demonstrates the successful
    immunization and immune response in a transgenic mouse of the
    present invention.

    Immunization of Mice
30   Keyhole limpet hemocyanin conjugated with greater
    than 400 dinitrophenyl groups per molecule (Calbiochem, La
    Jolla, California) (KLH-DNP) was alum precipitated according

to a previously published method (Practical Immunology, L.
Hudson and F.C. Hay, Blackwell Scientific (Pubs.), p. 9,
1980). Four hundred μg of alum precipitated KLH-DNP along
with 100 μg dimethyldioctadecyl Ammonium Bromide in 100 μL of

5   phosphate buffered saline (PBS) was injected intraperitoneally
into each mouse. Serum samples were collected six days later
by retro-orbital sinus bleeding.

Analysis of Human Antibody Reactivity in Serum

10   Antibody reactivity and specificity were assessed
using an indirect enzyme-linked immunosorbent assay (ELISA).
Several target antigens were tested to analyze antibody
induction by the immunogen. Keyhole limpet hemocyanin
(Calbiochem) was used to identify reactivity against the

15   protein component, bovine serum albumin-DNP for reactivity
against the hapten and/or modified amino groups, and KLH-DNP
for reactivity against the total immunogen. Human antibody
binding to antigen was detected by enzyme conjugates specific
for IgM and IgG sub-classes with no cross reactivity to mouse

20   immunoglobulin. Briefly, PVC microtiter plates were coated
with antigen drying overnight at 37°C of 5 μg/mL protein in
PBS. Serum samples diluted in PBS, 5% chicken serum, 0.5%
Tween-20 were incubated in the wells for 1 hour at room
temperature, followed by anti-human IgG Fc and IgG F(ab')-

25   horseradish peroxidase or anti-human IgM Fc-horseradish
peroxidase in the same diluent. After 1 hour at room
temperature enzyme activity was assessed by addition of ABTS
substrate (Sigma, St. Louis, Missouri) and read after 30
minutes at 415-490 nm.

30

Human Heavy Chain Participation in Immune Response in
Transgenic Mice

Figures 37A-37D illustrate the response of three
mouse littermates to immunization with KLH-DNP. Mouse number
1296 carried the human IgM and IgG unrearranged transgene and
was homozygous for mouse Ig heavy chain knockout. Mouse

5   number 1299 carried the transgene on a non-knockout
background, while mouse 1301 inherited neither of these sets
of genes. Mouse 1297, another littermate, carried the human
transgene and was hemizygous with respect to mouse heavy chain
knockout. It was included as a non-immunized control.

10   The results demonstrate that both human IgG and IgM
responses were developed to the hapten in the context of
conjugation to protein. Human IgM also developed to the KLH
molecule, but no significant levels of human IgG were present
at this time point. In pre-immunization serum samples from

15   the same mice, titers of human antibodies to the same target
antigens were insignificant.

                          EXAMPLE 20
         This example demonstrates the successful

20   immunization with a human antigen and immune response in a
transgenic mouse of the present invention, and provides data
demonstrating that nonrandom somatic mutation occurs in the
variable region sequences of the human transgene.

25   <u>Demonstration of antibody responses comprising human
immunoglobulin heavy chains against a human glycoprotein
antigen</u>

         Transgenic mice used for the experiment were
homozygous for functionally disrupted murine immunoglobulin

30   heavy chain loci produced by introduction of a transgene at
the joining (J) region (<u>supra</u>) resulting in the absence of
functional endogenous (murine) heavy chain production. The

transgenic mice also harbored at least one complete
unrearranged human heavy chain mini-locus transgene, (HC1,
supra), which included a single functional V_H gene (V_H251),
human μ constant region gene, and human γ1 constant region
5   gene. Transgenic mice shown to express human immunoglobulin
transgene products (supra) were selected for immunization with
a human antigen to demonstrate the capacity of the transgenic
mice to make an immune response against a human antigen
immunization. Three mice of the HC1-26 line and three mice of
10   the HC1-57 line (supra) were injected with human antigen.

One hundred μg of purified human carcinoembryonic
antigen (CEA) insolubilized on alum was injected in complete
Freund's adjuvant on Day 0, followed by further weekly
injections of alum-precipitated CEA in incomplete Freund's
15   adjuvant on Days 7, 14, 21, and 28. Serum samples were
collected by retro-orbital bleeding on each day prior to
injection of CEA. Equal volumes of serum were pooled from
each of the three mice in each group for analysis.

Titres of human μ chain-containing immunoglobulin
20   and human γ chain-containing immunoglobulin which bound to
human CEA immobilized on microtitre wells were determined by
ELISA assay. Results of the ELISA assays for human μ chain-
containing immunoglobulins and human γ chain-containing
immmunoglbulins are shown in Figs. 38 and 39, respectively.
25   Significant human μ chain Ig titres were detected for both
lines by Day 7 and were observed to rise until about Day 21.
For human γ chain Ig, significant titres were delayed, being
evident first for line HC1-57 at Day 14, and later for line
HC1-26 at Day 21. Titres for human γ chain Ig continued to
30   show an increase over time during the course of the
experiment. The observed human μ chain Ig response, followed
by a plateau, combined with a later geveloping γ chain

response which continues to rise is characteristic of the pattern seen with affinity maturation. Analysis of Day 21 samples showed lack of reactivity to an unrelated antigen, keyhole limpet hemocyanin (KLC), indicating that the antibody

5   response was directed against CEA in a specific manner.

These data indicate that animals transgenic for human unrearranged immunoglobulin gene loci: (1) can respond to a human antigen (e.g., the human glycoprotein, CEA), (2) can undergo isotype switching ("class switching) as

10 exemplified by the observed μ to γ class switch, and (3) exhibit characteristics of affinity maturation in their humoral immune responses. In general, these data indicate: (1) the human Ig transgenic mice have the ability to induce heterologous antibody production in response to a defined

15 antigen, (2) the capacity of a single transgene heavy chain variable region to respond to a defined antigen, (3) response kinetics over a time period typical of primary and secondary response development, (4) class switching of a transgene-encoded humoral immune response from IgM to IgG, and (5) the

20 capacity of transgenic animal to produce human-sequence antibodies against a human antigen.

Demonstration of somatic mutation in a human heavy chain transgene minilocus.

25   Line HC1-57 transgenic mice, containing multiple copies of the HC1 transgene, were bred with immunoglobulin heavy chain deletion mice to obtain mice that contain the HC1 transgene and contain disruptions at both alleles of the endogenous mouse heavy chain (supra). These mice express

30 human mu and gamma1 heavy chains together with mouse kappa and lambda light chains (supra). One of these mice was hyperimmunized against human carcinoembryonic antigen by

repeated intraperitoneal injections over the course of 1.5
months. This mouse was sacrificed and lymphoid cells isolated
from the spleen, inguinal and mesenteric lymph nodes, and
peyers patches. The cells were combined and total RNA
isolated. First strand cDNA was synthesized from the RNA and
used as a template for PCR amplification with the following 2
oligonucleotide primers:

149 5'-cta gct cga gtc caa gga gtc tgt gcc gag gtg cag ctg
(g/a/t/c)-3'

151 5'-ggc gct cga gtt cca cga cac cgt cac cgg ttc-3'

These primers specifically amplify VH251/gamma1 cDNA
sequences. The amplified sequences were digested with XhoI
and cloned into the vector pNN03. DNA sequence from the
inserts of 23 random clones is shown in Fig. 40; sequence
variations from germline sequence are indicated, dots indicate
sequence is identical to germline. Comparison of the cDNA
sequences with the germline sequence of the VH251 transgene
reveals that 3 of the clones are completely unmutated, while
the other 20 clones contain somatic mutations. One of the 3
non-mutated sequences is derived from an out-of-frame VDJ
joint. Observed somatic mutations at specific positions of
occur at similar frequencies and in similar distribution
patterns to those observed in human lymphocytes (Cai et al.
(1992) J. Exp. Med. 176: 1073, incorporated herein by
reference). The overall frequency of somatic mutations is
approximately 1%; however, the frequency goes up to about 5%
within CDR1, indicating selection for amino acid changes that
affect antigen binding. This demonstrates antigen driven
affinity maturation of the human heavy chain sequences.

EXAMPLE 21

This example demonstrates the successful formation
of a transgene by co-introduction of two separate
polynucleotides which recombine to form a complete human light
5   chain minilocus transgene.

Generation of an unrearranged light chain minilocus transgene
by co-injection of two overlapping DNA fragments

1.   Isolation of unrearranged functional V$_\kappa$ gene segments
10   vk65.3, vk65.5, vk65.8 and vk65.15

The V$_\kappa$ specific oligonucleotide, oligo-65 (5'-agg ttc
agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc agc-3'),
was used to probe a human placental genomic DNA library cloned
into the phage vector λEMBL3/SP6/T7 (Clonetech Laboratories,
15   Inc., Palo Alto, CA). DNA fragments containing V$_\kappa$ segments
from positive phage clones were subcloned into plasmid
vectors. Variable gene segments from the resulting clones are
sequenced, and clones that appear functional were selected.
Criteria for judging functionality include: open reading
20   frames, intact splice acceptor and donor sequences, and intact
recombination sequence. DNA sequences of 4 functional V$_\kappa$ gene
segments (vk65.3, vk65.5, vk65.8, and vk65.15) from 4
different plasmid clones isolated by this procedure are shown
in Figs. 41-44. The four plasmid clones, p65.3f, p65.5g1,
25   p65.8, and p65.15f, are described below.

(1 a) p65.3f

A 3 kb Xba fragment of phage clone λ65.3 was
subcloned into pUC19 so that the vector derived SalI site was
30   proximal to the 3' end of the insert and the vector derived
BamHI site 5'. The 3 kb BamHI/SalI insert of this clone was
subcloned into pGP1f to generate p65.3f.

(1 b) p65.5g1

  A 6.8 kb EcoRI fragment of phage clone λ65.5 was
subcloned into pGP1f so that the vector derived XhoI site is
proximal to the 5' end of the insert and the vector derived
5 SalI site 3'. The resulting plasmid is designated p65.5g1.

(1 c) p65.8

  A 6.5 kb HindIII fragment of phage clone λ65.8 was
cloned into pSP72 to generate p65.8.

10

(1 d) p65.15f

  A 10 kb EcoRI fragment of phage clone λ65.16 was
subcloned into pUC18 to generate the plasmid p65.15.3. The V_κ
gene segment within the plasmid insert was mapped to a 4.6 kb
15 EcoRI/HindIII subfragment, which was cloned into pGP1f. The
resulting clone, p65.15f, has unique XhoI and SalI sites
located at the respective 5' and 3' ends of the insert.

2. **pKV4**

20   The XhoI/SalI insert of p65.8 was cloned into the
XhoI site of p65.15f to generate the plasmid pKV2. The
XhoI/SalI insert of p65.5g1 was cloned into the XhoI site of
pKV2 to generate pKV3. The XhoI/SalI insert of pKV3 was
cloned into the XhoI site of p65.3f to generate the plasmid
25 pKV4. This plasmid contains a single 21 kb XhoI/SalI insert
that includes 4 functional V_κ gene segments. The entire insert
can also be excised with NotI.

3. **pKC1B**

30 (3 a) pKcor

  Two XhoI fragments derived from human genomic DNA
phage λ clones were subcloned into plasmid vectors. The

first, a 13 kb $J_\kappa 2$-$J_\kappa 5$/C_κ containing fragment, was treated with
Klenow enzyme and cloned into HindIII digested, Klenow
treated, plasmid pGP1d. A plasmid clone (pK-31) was selected
such that the 5' end of the insert is adjacent to the vector
derived ClaI site. The second XhoI fragment, a 7.4 kb piece
of DNA containing $J_\kappa 1$ was cloned into XhoI/SalI-digested pSP72,
such that the 3' insert XhoI site was destroyed by ligation to
the vector SalI site. The resulting clone, p36.2s, includes
an insert derived ClaI site 4.5 kb upstream of $J_\kappa 1$ and a
polylinker derived ClaI site downstream in place of the
naturally occurring XhoI site between $J_\kappa 1$ and $J_\kappa 2$. This clone
was digested with ClaI to release a 4.7 kb fragment which was
cloned into ClaI digested pK-31 in the correct 5' to 3'
orientation to generate a plasmid containing all 5 human J_κ
segments, the human intronic enhancer human C_κ, 4.5 kb of 5'
flanking sequence, and 9 kb of 3' flanking sequence. This
plasmid, pKcor, includes unique flanking XhoI and SalI sites
on the respective 5' and 3' sides of the insert.

(3 b) pKcorB

A 4 kb BamHI fragment containing the human 3' kappa
enhancer (Judde, J.-G. and Max, E.E. (1992) <u>Mol. Cell. Biol.</u>
<u>12</u>: 5206, incorporated herein by reference) was cloned into
pGP1f such that the 5' end is proximal to the vector XhoI
site. The resulting plasmid, p24Bf, was cut with XhoI and the
17.7 kb XhoI/SalI fragment of pKcor cloned into it in the same
orientation as the enhancer fragment. The resulting plasmid,
pKcorB, includes unique XhoI and SalI sites at the 5' and 3'
ends of the insert respectively.

(3 c) pKC1B

The XhoI/SalI insert of pKcorB was cloned into the
SalI site of p65.3f to generate the light-chain minilocus-
transgene plasmid pKC1B. This plasmid includes a single
functional human V_κ segment, all 5 human J_κ segments, the human
5   intronic enhancer, human C_κ, and the human 3' kappa enhancer.
The entire 25 kb insert can be isolated by NotI digestion.

4.   <u>Co4</u>

The two NotI inserts from plasmids pKV4 and pKC1B
10   were mixed at a concentration of 2.5 µg/ml each in
microinjection buffer, and co-injected into the pronuclei of
half day mouse embryos as described in previous examples.
Resulting transgenic animals contain transgene inserts
(designated Co4, product of the recombination shown in Fig.
15   45) in which the two fragments co-integrated. The 3' 3 kb of
the pKV4 insert and the 5'3 kb of the pKC1B insert are
identical. Some of the integration events will represent
homologous recombinations between the two fragments over the 3
kb of shared sequence. The Co4 locus will direct the
20   expression of a repertoire of human sequence light chains in a
transgenic mouse.

EXAMPLE 22

This example demonstrates the successful production
25   of a murine hybridoma clone secreting a monoclonal antibody
reactive with a specific immunogen, wherein the monoclonal
antibody comprises a human immunoglobulin chain encoded by a
human Ig transgene.

<u>Generation of Monoclonal Antibodies Incorporating Human Heavy</u>
30   <u>Chain Transgene Product</u>

1.   <u>Immunization of Mouse Harboring Human Heavy Chain</u>
    <u>Transgene</u>

A mouse containing a human heavy chain encoding transgene and homozygous for knockout (i.e., functional disruption) of the endogenous heavy chain locus (see, EXAMPLE 20, supra) was immunized with purified human CEA, and spleen

5 cells were subsequently harvested after a suitable immune response period. The murine spleen cells were fused with mouse myeloma cells to generate hybridomas using conventional techniques (see, Kohler and Milstein, Eur. J. Immunol., 6:511-519 (1976); Harlow and Lane, Antibodies: A Laboratory Manual,

10 Cold Spring Harbor, New York (1988)). The mouse used for immunization contained a human unrearranged heavy chain minilocus transgene which comprised a single functional V_H gene (V_{H251}), human D and J segments, human μ constant region, and human $\gamma1$ constant region genes. The transgenic line from

15 which it originated was designated HC1-57 (supra).

One hundred μg of purified human carcinoembryonic antigen (CEA) (Cyrstal Chem, Chicago, IL or Scripps Labs, San Diego, CA) insolubilized on alum was injected in complete Freund's adjuvant on Day 0, followed by further weekly

20 injections of alum-precipitated CEA in incomplete Freund's adjuvant on Days 7, 14, 21, and 28. An additional 20 μg of soluble CEA was administered intravenously on Day 83, followed by 50 μg alum-precipitated CEA in incomplete Freund's adjuvant on Day 92. Human heavy chain responses to CEA were confirmed

25 in serum samples prior to fusion of spleen cells with myleoma cells. The animal was sacrificed on Day 95, the spleen removed and fused with P3X63-Ag8.653 mouse myeloma cells (ATCC CRL 1580, American Type Culture Collection, Rockville, MD) using polyethylene glycol. Two weeks later, supernates from

30 fusion wells were screened for the presence of antibodies specifically reactive with CEA, and which contained human heavy chain μ or γ constant region epitopes by ELISA.

Briefly, purified human CEA was coated onto PVC microtitre plates at 2.5 μg/ml, and incubate with culture supernate diluted 1:4 or 1:5 in PBS, 0.5% Tween-20, 5% chicken serum. Plates were washed, followed by addition of horseradish

5 peroxidase-conjugated goat antiserum specific for human IgG Fc or rabbit antiserum specific for human IgM Fc5Mu (Jackson ImmunoResearch, West Grove, PA). Presence of conjugate bound to captured antibody was determined, after further washing, by the addition of ABTS substrate. Two independent fusion wells

10 were found to contain antibody with substantial binding to CEA. After cloning, both hybridomas were found to be positive for the presence of human μ chain and murine κ chain by ELISA. No mouse IgG or IgM were detected using similar assays.

Subcloning of the two independent parent hybridomas

15 resulted in two clones, designated 92-09A-4F7-A5-2 and 92-09A-1D7-1-7-1. Both lines were deposited with the ATCC Patent Culture Depository under the Budapest Treaty and were assigned ATCC Designation HB 11307 and HB 11308, respectively. Culture supernatants from these cell lines were assessed for

20 specificity by testing for reactivity to several purified target proteins using ELISA. As shown in Fig. 46, ELISA assays for determining the reactivity of the monoclonal antibodies to various antigens demonstrate that only CEA and the CEA-related antigen NCA-2 show significant reactivity,

25 indicating the development of a restricted reactivity for the variable regions of the heterohybrid immunoglobulin molecules.

EXAMPLE 23

This example demonstrates that a rearranged human

30 VDJ gene encoded by a human Ig minilocus transgene may be transcribed as a transcript which includes an endogenous Ig

constant region gene, for example by the mechanism of trans-
switching, to encode a chimeric human/mouse Ig chain.

Identification of Trans-Switch Transcripts Encoding Chimeric
Human-Mouse Heavy Chains

5   RNA was isolated from a hyperimmunized HC1 line 57
transgenic mouse homozygous for the endogenous heavy chain J
segment deletion (supra). cDNA was synthesized according to
Taylor et al. (1993) Nucleic Acids Res. 20: 6287, incorporated
herein by reference, and amplified by PCR using the following
10   two primers:

          o-149 (human V_{H251}):
     5'-CTA GCT CGA GTC CAA GGA GTC TGT GCC GAG GTG CAG CTG (G,A,T,C)-3'
          o-249 (mouse gamma):
15   5'-GGC GCT CGA GCT GGA CAG GG(A/C) TCC A(G/T)A GTT CCA-3'

          Oligonucleotide o-149 is specific for the HC1-
encoded variable gene segment V_{H251}, while o-249 hybridizes to
both mouse and human gamma sequences with the following order
20   of specificities:
mouse γ1 = mouse γ2b = mouse γ3 > mouse γ2a >> human γ1.
DNA sequences from 10 randomly chosen clones generated from
the PCR products was determined and is shown in Fig. 47. Two
clones comprised human VDJ and mouse γ1; four clones comprised
25   human VDJ and mouse γ2b; and four clones comprised human VDJ
and mouse γ3. These results indicate that in a fraction of
the transgenic B cells, the transgene-encoded human VDJ
recombined into the endogenous murine heavy chain locus by
class switching or an analogous recombination.

30

EXAMPLE 24

This example describes a method for screening a pool
of hybridomas to discriminate clones which encode chimeric
human/mouse Ig chains from clones which encode and express a
human Ig chain. For example, in a pool of hybridoma clones
5   made from a transgenic mouse comprising a human Ig heavy chain
transgene and homozygous for a J region-disrupted endogenous
heavy chain locus, hybridoma clones encoding trans-switched
human VDJ-murine constant region heavy chains may be
identified and separated from hybridoma clones expressing
10   human VDJ-human constant region heavy chains.

Sceening Hybridomas to Eliminate Chimeric Ig Chains

The screening process involves two stages, which may
be conducted singly or optionally in combination: (1) a
preliminary ELISA-based screen, and (2) a secondary molecular
15   characterization of candidate hybridomas. Preferably, a
preliminary ELISA-based screen is used for initial
identification of candidate hybridomas which express a human
VDJ region and a human constant region.

Hybridomas that show positive reactivity with the
20   antigen (e.g., the immunogen used to elicit the antibody
response in the transgenic mouse) are tested using a panel of
monoclonal antibodies that specifically react with mouse μ, γ,
κ, and λ, and human μ, γ, and κ. Only hybridomas that are
positive for human heavy and light chains, as well as negative
25   for mouse chains, are identified as candidate hybridomas that
express human immunoglobulin chains. Thus, candidate
hybridomas are shown to have reactivity with specific antigen
and to possess epitopes characteristic of a human constant
region.

30   RNA is isolated from candidate hybridomas and used
to synthesize first strand cDNA. The first strand cDNA is
then ligated to a unique single-stranded oligonucleotide of

predetermined sequence (oligo-X) using RNA ligase (which
ligates single-stranded DNA). The ligated cDNA is then
amplified in two reactions by PCR using two sets of
oligonucleotide primers. Set H (heavy chain) includes an
oligo that specifically anneals to either human μ or human γ1
(depending on the results of the ELISA) and an oligo that
anneals to the oligo-X sequence. This prevents bias against
detection of particular V segments, including mouse V segments
that may have trans-rearranged into the human minilocus. A
second set of primers, Set L (light chain), includes an oligo
that specifically anneals to human κ and an oligo that anneals
specifically to oligo-X. The PCR products are molecularly
cloned and the DNA sequence of several are determined to
ascertain whether the hybridoma is producing a unique human
antibody on the basis of sequence comparison to human and
murine Ig sequences.

                            EXAMPLE 25

        This example demonstrates production of a transgenic
mouse harboring a human light chain (κ) minilocus.
<u>Human κ Minilocus transgenic mice</u>
<u>KC1</u>

        A 13 kb XhoI Jκ2-Kκ containing fragment from a phage
clone (isolated from a human genomic DNA phage library by
hybridization to a κ specific oligonucleotide, e.g., *supra*)
was treated with Klenow enzyme and cloned into the Klenow
treated HindIII site of pGP1d to produce pK-31. This
destroyed the insert XhoI sites and positioned the unique
polylinker derived XhoI site at the 5' end next to Jκ2. A
unique polylinker derived ClaI site is located between this
XhoI site and the inset sequences, while a unique polylinker
derived SalI site is located at the 3' end of the insert. A

7.5 kb XhoI fragment, containing Jĸ1 and upstream sequences,
was also isolated from a human genomic DNA phage clone
(isolated from a human genomic DNA phage library by
hybridization to a ĸ specific oligonucleotide, e.g. *supra*).

5　This 7.5 kb XhoI fragment was cloned into the SalI site of
pSP72 (Promega, Madison, Wisconsin), thus destroying both XhoI
sites and positioning a polylinker ClaI site 3' of Jĸ1.
Digestion of the resulting clone with ClaI released a 4.7 kb
fragment containing Jk1 and 4.5 kb of upstream sequences.

10　This 4.7 kb fragment was cloned into the ClaI site of pK-31 to
create pKcor. The remaining unique 5' XhoI site is derived
from polylinker sequences. A 6.5 kb XhoI/SalI DNA fragment
containing the unrearranged human VĸIII gene segment 65.8
(plasmid p65.8, EXAMPLE 21) was cloned into the XhoI site of

15　pKcor to generate the plasmid pKC1. The NotI insert of pKC1
was microinjected into 1/2 day mouse embryos to generate
transgenic mice. Two independent pKC1 derived transgenic
lines were established and used to breed mice containing both
heavy and light chain miniloci. These lines, KC1-673 and KC1-

20　674, were estimated by Southern blot hybridization to contain
integrations of approximately 1 and 10-20 copies of the
transgenes respectively.

　　KC1e

25　　　　　The plasmid pMHE1 (EXAMPLES 13 and 18) was digested
with BamHI and HindIII to excise the 2.3 kb insert containing
both the mouse and human heavy chain J-μ intronic enhancers.
This fragment was Klenow treated, ligated to SalI linkers (New
England Biolabs, Beverly, Massachusetts), and cloned into the

30　unique 3' SalI site of pKC1 to generate the plasmid pKC1e.
The NotI insert of pKC1e was microinjected into 1/2 day mouse
embryos to generate transgenic mice. Four independent pKC1e

derived transgenic lines were established and used to breed
mice containing both heavy and light chain miniloci. These
lines, KCle-1399, KCle-1403, KCle-1527, and KCle-1536, were
estimated by Southern blot hybridization to contain
5   integrations of approximately 20-50, 5-10, 1-5, and 3-5 copies
of the transgene, respectively.

pKC2

     A 6.8 kb XhoI/SalI DNA fragment containing the
10   unrearranged human VκIII gene segment 65.5 (plasmid p65.5g1,
EXAMPLE 21) was cloned into the unique 5' XhoI site of pKC1 to
generate the plasmid pKC2. This minilocus transgene contains
two different functional VκIII gene segments. The NotI insert
of pKC2 was microinjected into 1/2 day mouse embryos to
15   generate transgenic mice. Five independent pKC2 derived
transgenic lines were established and used to breed mice
containing both heavy and light chain miniloci. These lines,
KC2-1573, KC2-1579, KC2-1588, KC2-1608, and KC2-1610, were
estimated by Southern blot hybridization to contain
20   integrations of approximately 1-5, 10-50, 1-5, 50-100, and 5-
20 copies of the transgene, respectively.

EXAMPLE 26

     This example shows that transgenic mice bearing the
25   human κ transgene can make an antigen-induced antibody
response forming antibodies comprising a functional human κ
chain.

Antibody Responses Associated with Human Ig κ Light Chain

     A transgenic mouse containing the HC1-57 human heavy
30   chain and KCle human κ transgenes was immunized with purified
human soluble CD4 (a human glycoprotein antigen). Twenty μg
of purified human CD4 (NEN Research products, Westwood, MA)

insolublized by conjugation to polystyrene latex particles
(Polysciences, Warrington, PA) was injected intraperitoneally
in saline with dimethyldioctadecyl ammonium bromide
(Calbiochem, San Diego, CA) on Day 0, followed by further
5   injections on Day 20 and Day 34.

        Retro-orbital bleeds were taken on Days 25 and 40,
and screened for the presence of antibodies to CD4, containing
human IgM or human IgG heavy chain by ELISA. Briefly,
purified human CD4 was coated onto PVC microtitre plates at
10   2.5 μg/ml and incubated with culture supernate diluted 1:4/1:5
in PBS, 0.5% Tween-20, 5% chicken serum. Plates were washed,
followed by addition of horseradish peroxidase-conjugated goat
antiserum specific for human IgG Fc or rabbit antiserum
specific for human IgM Fc5Mu (Jackson ImmunoResearch, Westr
15   Grove, PA). Presence of conjugate bound to captured antibody
was determined after further washing by addition of ABTS
substrate. Human μ reactive with antigen was detected in both
bleeds, while there was essentially undetectable γ reactivity.
The Day 40 sample was also tested for antigen-reactive human κ
20   chain using the same assay with goat anti-human κ peroxidase
conjugate (Sigma, St. Louis, MO). CD4-binding κ reactivity
was detected at this time point. The assay results are shown
in Fig. 48.

                          EXAMPLE 27
25   This example shows the successful generation of mice
which are homozygous for functionally disrupted murine heavy
and light chain loci (heavy chain and κ chain loci) and which
concomitantly harbor a human heavy chain transgene and a human
light chain transgene capable of productively rearranging to
30   encode functional human heavy chains and functional human
light chains. Such mice are termed "0011" mice, indicating by
the two 0's in the first two digits that the mice lack

functional heavy and light chain loci and indicating by the
1's in the second two digits that the mice are hemizygous for
a human heavy chain transgene and a human light chain
transgene. This example shows that such 0011 mice are capable
5   of making a specific antibody response to a predetermined
antigen, and that such an antibody response can involve
isotype switching.

0011/0012 Mice: Endogenous Ig Knockout + Human Ig Transgenes

Mice which were homozygous for a functionally
10   disrupted endogenous heavy chain locus lacking a functional J_H
region (designated JHD++ or JHΔ++) and also harboring the
human HC1 transgene, such as the HC1-26 transgenic mouse line
described *supra*, were interbred with mice homozygous for a
functionally disrupted endogenous kappa chain locus lacking a
15   functional J_H region (designated here as JKD++ or JKΔ++; see
Example 9) to produce mice homozygous for functionally
disrupted heavy chain and kappa chain loci (heavy chain/kappa
chain knockouts), designated as JHD++/JKD++ and containing a
HC1 transgene. Such mice were produced by interbreeding and
20   selected on the basis of genotype as evaluated by Southern
blot of genomic DNA. These mice, designated HC1-
26+/JKD++/JHD++ mice, were interbred with mice harboring a
human kappa chain transgene (lines KC2-1610, KC1e-1399, and
KC1e-1527; see Example 25), and Southern blot analysis of
25   genomic DNA was used to identify offspring mice homozygous for
functionally disrupted heavy and light chain loci and also
hemizygous for the HC1 transgene and the KC2 or KC1e
transgene. Such mice are designated by numbers and were
identified as to their genotype, with the following
30   abbreviations: HC1-26+ indicates hemizygosity for the HC1-26
line human heavy chain minilocus transgene integration; JHD++
indicates homozygosity for J_H knockout; JKD++ indicates

homozygosity for J_κ knockout; KC2-1610+ indicates hemizygosity

for a KC2 human κ transgene integrated as in line KC2-1610;

KC1e-1527+ indicates hemizygosity for a KC1e human κ transgene

integrated as in line KC1e-1527; KC1e-1399+ indicates

5   hemizygosity for a KC1e human κ transgene integrated as in

line KC1e-1399.

The resultant individual offspring were each given a

numerical designation (e.g., 6295, 6907, etc.) and each was

evaluated for the presence of J_H knockout alleles, J_κ knockout

10 alleles, HC1-26 transgene, and κ transgene (KC2 or KC1e) and

determined to be either hemizygous (+) or homozygous (++) at

each locus. Table 10 shows the number designation, sex, and

genotypes of several of the offspring mice.

Table 10

ID No.	Sex	Ig Code	Genotype
6295	M	0011	HC1-26+;JHD++;JKD++;KC2-1610+
6907	M	0011	HC1-26+;JHD++;JKD++;KC1e-1527+
7086	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7088	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7397	F	0011	HC1-26+;JHD++;JKD++;KC1e-1527+
7494	F	0012	HC1-26+;JHD++;JKD++;KC2-1610++
7497	M	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7648	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7649	F	0012	HC1-26+;JHD++;JKD++;KC2-1610++
7654	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7655	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7839	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7656	F	0001	HC1-26-;JHD++;JKD++;KC2-1610+
7777	F	1100	Co1-2141-;JHD+;JKD+

We removed spleens from three 6 week old female

mice. Mouse # 7655 was determined by Southern blot

hybridization to be hemizygous for the HC1 (line 26) and KC2
(line 1610) transgene integrations, and homozygous for the JHΔ
and JκΔ targeted deletions of the mouse μ and κJ regions.
Mouse #7656 was determined by Southern blot hybridization to

5   be hemizygous for the KC2 (line 1610) transgene integration
and homozygous for the JHΔ and JκΔ targeted deletions of the
mouse μ and κJ regions. Mouse # 7777 was determined by
Souther blot hybridization to be hemizygous for the JHΔ and
JκΔ targeted deletions of the mouse μ and κJ regions. Because

10 of the recessive nature of these deletions, this mouse should
be phenotypically wild-type.

Expression of Endogenous Iq Chains in 0011 Mice

FACS analysis using a panel of antibodies reactive

15 with either human μ, mouse μ, hman κ, mouse κ, or mouse λ was
used to sort lymphocytes explanted from (1) a wildtype mouse
(7777), (2) a 0001 mouse homozygous for heavy chain and kappa
knockout alleles and harboring a human light chain transgene
(7656), and (3) a 0011 mouse homozygous for heavy chain and

20 kappa knockout alleles and harboring a human light chain
transgene and a human heavy chain transgene (7655).

We prepared single cell suspensions from spleen and
lysed the red cells with NH₄Cl, as described by Mishell and
Shiigi (Mishell, B.B. & Shiigi, S.M. (eds) Selected Methods in

25 Cellular Immunology. W.H. Freeman & Co., New York, 1980).
The lymphocytes are stained with the following reagents:
propidium iodide (Molecular Probes, Eugene, OR), FITC
conjugated anti-human IgM (clone G20-127; Pharmingen, San
Diego, CA), FITC conjugated anti-mouse IgM (clone R6-60.2;

30 Pharmingen, San Diego, CA), phycoerythrin conjugated anti-
human Igκ (clone HP6062; CalTag, South San Francisco, CA),
FITC conjugated anti-mouse Igλ (clone R26-46; Pharmingen, San

Diego, CA) FITC conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, CA), and Cy-Chrome conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, CA). We analyzed the stained cells using a FACScan flow cytometer and

5 LYSIS II software (Becton Dickinson, San Jose, CA). Macrophages and residual red cells are excluded by gating on forward and side scatter. Dead cells are excluded by gating out propidium iodide positive cells. The flow cytometric data in Figs. 49 and 50 confirms the Southern blot hybridization

10 data and demonstrates that mouse #7655 expresses both human μ and human κ and relatively little if any mouse μ or mouse κ. Nevertheless a significant fraction of the B cells (about 70-80%) appear to express hybrid Ig receptors consisting of human heavy and mouse λ light chains.

15   Fig. 49 shows the relative distribution of B cells expressing human μ or mouse μ on the cell surface; 0011 mouse (7655) lymphocytes are positive for human μ but relatively lack mouse μ; 0001 mouse (7656) lymphocytes do not express much human μ or mouse μ; wildtype mouse (7777) lymphocytes

20 express mouse μ but lack human μ.

        Fig. 50 shows the relative distribution of B cells expressing human κ or mouse κ on the cell surface; 0011 mouse (7655) lymphocytes are positive for human κ but relatively lack mouse κ; 0001 mouse (7656) lymphocytes do not express

25 much human κ or mouse κ; wildtype mouse (7777) lymphocytes express mouse κ but lack human κ.

        Fig. 51 shows the relative distribution of B cells expressing mouse λ on the cell surface; 0011 mouse (7655) lymphocytes are positive for mouse λ; 0001 mouse (7656)

30 lymphocytes do not express significant mouse λ; wildtype mouse (7777) lymphocytes express mouse λ but at a relatively lower level than the 0011 mouse (7655).

Fig. 52 shows the relative distribution of B cells
positive for endogenous mouse λ as compared to human κ
(transgene-encoded). The upper left panel shows the results
of cells from a wildtype mouse possessing functional

5   endogenous heavy and light chain alleles and lacking human
transgene(s); the cells are positive for mouse lambda. The
upper right panel shows cells from a mouse (#5822) having a κ
knockout background (JKD++) and harboring the human κ
transgene intergration of the KC1e-1399 line; the cells are

10 positive for human κ or mouse λ in roughly proportional
amounts. The lower left panel shows cells from a mouse
(#7132) having a κ knockout background (JKD++) and harboring
the human κ transgene intergration of the KC2-1610 line; more
cells are positive for mouse λ than for human κ, possibly

15 indicating that the KC2-1610 transgene integration is less
efficient than the KC1e-1399 transgene integration. The lower
right panel shows cells from a mouse harboring a human κ
minilocus transgene (KCo4) and lacking a functional endogenous
murine κ allele. The data presented in Fig. 52 also

20 demonstrates the variability of phenotypic expression between
transgenes. Such variability indicates the desirability of
selecting for individual transgenes and/or transgenic lines
which express one or more desired phenotypic features
resulting from the integrated transgene (e.g., isotype

25 switching, high level expression, low murine Ig background).
Generally, single or multiple transgene species (e.g., pKC1e,
pKC2, KCo4) are employed separately to form multiple
individual transgenic lines differing by: (1) transgene, (2)
site(s) of transgene integration, and/or (3) genetic

30 background. Individual transgenic lines are examined for
desired parameters, such as: (1) capability to mount an immune
respone to a predetermined antigen, (2) frequency of isotype

switching within transgene-encoded constant regions and/or
frequency of trans-switching to endogenous (e.g., murine) Ig
constant region genes, (3) expression level of transgene-
encoded immmunoglobulin chains and antibodies, (4) expression

5   level of endogenous (e.g., murine) immunoglobulin
immunoglobulin sequences, and (5) frequency of productive VDJ
and VJ rearrangement. Typically, the transgenic lines which
produce the largest concentrations of transgene-encoded (e.g.,
human) immunoglobulin chains are selected; preferably, the

10 selected lines produce about at least 40 μg/ml of transgene-
encoded heavy chain (e.g., human μ or human γ) in the serum of
the transgenic animal and/or about at least 100 μg/ml of
transgene-encoded light chain (e.g., human κ).

          Mice were examined for their expression of human and

15 murine immmunoglobulin chains in their unimmunized serum and
in their serum following immunization with a specific antigen,
human CD4. Fig. 53 shows the relative expression of human μ,
human γ, murine μ, murine γ, human κ, murine κ, and murine λ
chains present in the serum of four separate unimmunized 0011

20 mice of various genotypes (nt = not tested); human κ
predominates as the most abundant light chain, and human μ and
murine γ (putatively a product of trans-switching) are the
most abundant heavy chains, with variability between lines
present, indicating the utility of a selection step to

25 identify advantageous genotypic combinations that minimize
expression of murine chains while allowing expression of human
chains. Mice #6907 and 7088 show isotype switching (cis-
switching within the transgene) from human μ to human γ.

          Fig. 54 shows serum immunoglobulin chain levels for

30 human μ (huμ), human γ (huγ), human κ (huκ), murine μ (msμ),
murine γ (msγ), murine κ (msκ), and murine λ (msλ) in mice of
the various 0011 genotypes.

Specific Antibody Response in 0011 Mice

An 0011 mouse (#6295) was immunized with an
immunogenic dose of human CD4 according to the following
immunization schedule: Day 0, intraperitoneal injection of 100

5   μl of CD4 mouse immune serum; Day 1, inject 20 μg of human CD4
(American Bio-Tech) on latex beads with DDA in 100 μl; Day 15
inject 20 μg of human CD4 (American Bio-Tech) on latex beads
with DDA in 100 μl; Day 29 inject 20 μg of human CD4 (American
Bio-Tech) on latex beads with DDA in 100 μl; Day 43 inject 20

10   μg of human CD4 (American Bio-Tech) on latex beads with DDA in
100 μl.

Fig. 55 shows the relative antibody response to CD4
immunization at 3 weeks and 7 weeks demonstrating the presence
of human μ, human κ, and human γ chains in the anti-CD4

15   response. Human γ chains are present at significantly
increased abundance in the 7 week serum, indicating that cis-
switching within the heavy chain transgene (isotype switching)
is occurring in a temporal relationship similar to that of
isotype switching in a wildtype animal.

20   Fig. 56 shows a schematic compilation of various
human heavy chain and light chain transgenes.

                              EXAMPLE 28

This example provides for the targeted knockout of

25   the murine λ light chain locus.

Targeted Inactivation of the Murine Lambda Light Chain Locus

Unlike the Ig heavy and kappa light chain loci, the
murine VλJλ and Cλ gene segments are not grouped into 3
families arranged in a 5' to 3' array, but instead are

30   interspersed. The most 5' portion consists of two V segments
(Vλ2 and VλX) which are followed, proceeding in a 3'
direction, by two constant region exons, each associated with

its own J segment (Jλ2Cλ2 and the pseudogene Jλ4Cλ4). Next is

the most extensively used V segment (Vλ1) which is followed by

the second cluster of constant region exons (Jλ3Cλ3 and

Jλ1Cλ1,). Overall the locus spans approximate 200 kb, with

5   intervals of ⌐20-90 kb between the two clusters.

Expression of the lambda locus involves

rearrangement of Vλ2 or VλX predominantly to Jλ2 and only

rarely further 3' to Jλ3 or Jλ1. Vλ1 can recombine with both

Jλ3 and Jλ1. Thus the lambda locus can be mutated in order to

10   fully eliminate recombination and expression of the locus.

The distance between the two lambda gene clusters

makes it difficult to inactivate expression of the locus via

the generation of a single compact targeted deletion, as was

used in inactivating the murine Ig heavy and kappa light chain

15   loci. Instead, a small single deletion which would eliminate

expression lambda light chains spans approximately 120 kb,

extending from Jλ2Cλ2 to Jλ1Cλ1 (Fig. 57). This removes all

of the lambda constant region exons as well as the Vλ1 gene

segment, ensuring inactivation of the locus.

20   Replacement type targeting vectors (Thomas and

Capecchi (1987) op.cit) are constructed in which the deleted

120 kb is replaced with the selectable marker gene, *neo*, in a

PGK expression cassette. The marker is embedded within

genomic lambda sequences flanking the deletion to provide

25   homology to the lambda locus and can also contain the HSV-*tk*

gene, at the end of one of the regions of homology, to allow

for enrichment for cells which have homologously integrated

the vectors. Lambda locus genomic clone sequences are

obtained by screening of a strain 129/Sv genomic phage library

30   isogenic to the ES line being targeted, since the use of

targeting vectors isogenic to the chromosomal DNA being

targeted has been reported to enhance the efficiency of

homologous recombination. Targeting vectors are constructed
which differ in their lengths of homology to the lambda locus.
The first vector (vector 1 in Fig. 58) contains the marker
gene flanked by total of approximately 8-12 kb of lambda locus

5   sequences. For targeting events in which replacement vectors
mediate addition or detection of a few kb of DNA this has been
demonstrated to be a more than sufficient extent of homology
(Hasty et al. (1991) op.cit; Thomas et al.(1992) op.cit.).
Vectors with an additional approximately 40-60 kb of flanking

10 lambda sequence are also constructed (vector 2 in Fig. 58).
Human Ig miniloci of at least 80 kb are routinely cloned and
propagated in the plasmid vector pGP1 (Taylor et al. (1993)
op.cit).

       An alternative approach for inactivation of the

15 lambda locus employs two independent mutations, for example
mutations of the two constant region clusters or of the two V
region loci, in the same ES cell. Since both constant regions
are each contained within ~6 kb of DNA, whereas one of the V
loci spans ~19 kb, targeting vectors are constructed to

20 independently delete the Jλ2Cλ2/Jλ4Cλ4 and the Jλ3Cλ3/Jλ1Cλ1
loci. As shown in Fig. 58, each vector consists of a
selectable marker (e.g., neo or pac) in a PGK expression
cassette, surrounded by a total of ~8-12 kb of lambda locus
genomic DNA blanking each deletion. The HSV-tk gene can be

25 added to the targeting vectors to enrich for homologous
recombination events by positive-negative selection. ES cells
are targeted sequentially with the two vectors, such that
clones are generated which carry a deletion of one of the
constant region loci; these clones are then targeted

30 sequentially with the two vectors, such that clones will be
generated which carry a deletion of one of the constant region
loci, and these clones are then targeted to generate a

deletion of the remaining functional constant region cluster.
Since both targeting events are thus being directed to the
same cell, it is preferable to use a different selectable
marker for the two targetings. In the schematic example shown

5   in Fig. 58, one of the vectors contains the neo gene and the
other the pac (puromycin N-acetyl transferase) gene. A third
potential dominant selectable marker is the hyg (hygromycin
phosphotransferase) gene. Both the pac and hyg genes can be
been inserted into the PGK expression construct successfully

10 used for targeting the neo gene into the Ig heavy and kappa
light chain loci. Since the two lambda constant region
clusters are tightly linked, it is important that the two
mutations reside on the same chromosome. There preferably is
a 50% probability of mutating the same allele by two

15 independent targeting events, and linkage of the mutations is
established by their co-segregation during breeding of
chimeras derived from the doubly targeted ES cells.

20   EXAMPLE 29
         This example provides for the targeted knockout of
the murine heavy chain locus.
<u>Targeted Inactivation of the Murine Heavy Chain Locus</u>
         A homologous recombination gene targeting transgene

25 having the structure shown in Fig. 59 is used to delete at
least one and preferably substantially all of the murine heavy
chain locus constant region genes by gene targeting in ES
cells. Fig. 59 shows a general schematic diagram of a
targeting transgene. Segment (a) is a cloned genomic DNA

30 sequence located upstream of the constant region gene(s) to be
deleted (i.e, proximal to the J_H genes); segment (b) comprises
a positive selection marker, such as pgk-neo; segment (c) is a

cloned genomic DNA sequence located downstream of the constant

region gene(s) to be deleted (i.e, distal to the constan

region gene(s) and and J_H genes); and segment (d), which is

optional, comprises a negative selection marker gene (e.g.,

5   HSV-tk). Fig. 60 shows a map of the murine heavy chain locus

as taken from Immunoglobulin Genes, Honjo, T, Alt, FW, and

Rabbits TH (eds.) Academic Press, NY (1989) p. 129.

A targeting transgene having a structure according

to Fig. 59, wherein: (1) the (a) segment is the 11.5 kb insert

10   of clone JH8.1 (Chen et al. (1993) Int. Immunol. 5: 647) or an

equivalent portion comprising about at least 1-4 kb of

sequence located upstream of the murine Cμ gene, (2) the (b)

segment is pgk-neo as described supra, (3) the (c) segment

comprises the 1674 bp sequence shown in Fig. 61 or a 4-6 kb

15   insert isolated from a phage clone of the mouse Cα gene

isolated by screening a mouse genomic clone library with the

end-labeled oligonucleotide having the sequence:

5'-gtg ttg cgt gta tca gct gaa acc tgg aaa cag ggt gac cag-3'

and (4) the (d) segment comprises the HSV-tk expression

20   cassette described supra.

Alternatively, a stepwise deletion of one or more

heavy chain constant region genes is performed wherein a first

targeting transgene comprises homology regions, i.e., segments

(a) and (c), homologous to sequences flanking a constant

25   region gene or genes, a first species of positive selection

marker gene (pgk-neo), and an HSV-tk negative selection

marker. Thus, the (a) segment can comprise a sequence of at

least about 1-4 kb and homologous to a region located upstream

of Cγ3 and the (c) segment can comprise a sequence of at least

30   about 1-4 kb and homologous to a region located upstream of

Cγ2a. This targeting transgene deletes the Cγ3, Cγ1, Cγ2b,

and Cγ2a genes. This first targeting transgene is introduced

into ES cells and correctly targeted recombinants are selected
(e.g., with G418), producing a correctly targeted C region
deletion. Negative selection for loss of the HSV-*tk* cassette
is then performed (e.g., with ganciclovir or FIAU). The

5   resultant correctly targeted first round C deletion
recombinants have a heavy chain locus lacking the Cγ3, Cγ1,
Cγ2b, and Cγ2a genes.

A second targeting transgene comprises homology
regions, i.e., segments (a) and (c), homologous to sequences

10   flanking a constant region gene or genes, a second species of
positive selection marker gene different that the first
species (e.g., gpt or pac), and an HSV-*tk* negative selection
marker. Thus, the (a) segment can comprise a sequence of at
least about 1-4 kb and homologous to a region located upstream

15   of Cε and the (c) segment can comprise a sequence of at least
about 1-4 kb and homologous to a region located upstream of
Cα. This targeting transgene deletes the Cε and Cα genes.

This second targeting transgene is introduced into
the correctly targeted C-region recombinant ES cells obtained

20   from the first targeting event. Cells which are correctly
targeted for the second knockout event (i.e., by homologous
recombination with the second targeting transgene) are
selected for with a selection drug that is specific for the
second species of positive selection marker gene (e.g.,

25   mycophenolic acid to select for gpt; puromycin to select for
pac). Negative selection for loss of the HSV-*tk* cassette is
then performed (e.g., with ganciclovir or FIAU). These
resultant correctly targeted second round C region
recombinants have a heavy chain locus lacking the Cγ3, Cγ1,

30   Cγ2b, Cγ2a, Cε, and Cα genes.

Correctly targeted first-round or second-round
recombinant ES cells lacking one or more C region genes are

used for blastocyst injections as described (*supra*) and
chimeric mice are produced. Germline transmission of the
targeted heavy chain alleles is established, and breeding of
the resultant founder mice is performed to generate mice
5   homozygous for C-region knockouts. Such C-region knockout
mice have several advantages as compared to J_H knockout mice;
for one example, C-region knockout mice have diminished
ability (or completely lack the ability) to undergo trans-
switching between a human heavy chain transgene and an
10   endogenous heavy chain locus constant region, thus reducing
the frequency of chimeric human/mouse heavy chains in the
transgenic mouse. Knockout of the murine gamma genes is
preferred, although μ and delta are frequently also deleted by
homologous targeting. C-region knockout can be done in
15   conjunction with other targeted lesions int he endogenous
murine heavy chain locus; a C-region deletion can be combined
with a J_H knockout to preclude productive VDJ rearrangement of
the murine heavy chain locus and to preclude or reduce trans-
switching between a human heavy chain transgene and the murine
20   heavy chain locus, among others. For some embodiments, it may
be desirable to produce mice which specifically lack one or
more C-region genes of the endogenous heavy chain locus, but
which retain certain other C-region genes; for example, it may
be preferable to retain the murine $C\alpha$ gene to allow to
25   production of chimeric human/mouse IgA by trans-switching, if
such IgA confers an advantageous phenotype and does not
substantially interfere with the desired utility of the mice.

                            EXAMPLE 30

30   This example demonstrates _ex vivo_ depletion of
lymphocytes expressing an endogenous (murine) immunoglobulin
from a lymphocyte sample obtained from a transgenic mouse

harboring a human transgene. The lymphocytes expressing murine Ig are selectively depleted by specific binding to an anti-murine immunoglobulin antibody that lacks substantial binding to human immunoglobulins encoded by the transgene(s).

5   Ex Vivo Depletion of Murine Ig-Expressing B-cells

A mouse homozygous for a human heavy chain minilocus transgene (HC2) and a human light chain minilocus transgene (KCo4) is bred with a C57BL/6 (B6) inbred mouse to obtain 2211 mice (i.e., mice which: are homozygous for a functional

10 endogenous murine heavy chain locus, are homozygous for a functional endogenous murine light chain locus, and which possess one copy of a human heavy chain transgene and one copy of a human light chain transgene). Such 2211 mice also express B6 major and minor histocompatibility antigens. These

15 mice are primed with an immunogenic dose of an antigen, and after approximately one week spleen cells are isolated. B cells positive for murine Ig are removed by solid phase-coupled antibody-dependent cell separation according to standard methods (Wysocki et al. (1978) Proc. Natl. Acad. Sci.

20 (U.S.A.) 75: 2844; MACS magnetic cell sorting, Miltenyi Biotec Inc., Sunnyvale, CA), followed by antibody-dependent complement-mediated cell lysis (Selected Methods in Cellular Immunology, Mishell BB and Shiigi SM (eds.), W.H. Freeman and Company, New York, 1980, pp.211-212) to substantially remove

25 residual cells positive for murine Ig. The remaining cells in the depleted sample (e.g., T cells, B cells positive for human Ig) are injected i.v., preferably together with additional anti-murine Ig antibody to deplete arising B cells, into a SCID/B6 or RAG/B6 mouse. The reconstitued mouse is then

30 further immunized for the antigen to obtain antibody and affinity matured cells for producing hybridoma clones.

EXAMPLE 31

Production of Fully Human Antibodies in Somatic Chimeras

A method is described for producing fully human antibodies in somatic chimeric mice. These mice are generated by introduction of embryonic stem (ES) cells, carrying human immunoglobulin (Ig) heavy and light chain transgenes and lacking functional murine Ig heavy and kappa light chain genes, into blastocysts from RAG-1 or RAG-2 deficient mice.

RAG-1 and RAG-2 deficient mice (Mombaerts et al. (1992) Cell 68: 869; Shinkai et al. (1992) Cell 68: 855) lack murine B and T cells due to an inability to initiate VDJ rearrangement and to assemble the gene segments encoding Igs and T cell receptors (TCR). This defect in B and T cell production can be complemented by injection of wild-type ES cells into blastocysts derived from RAG-2 deficient animals. The resulting chimeric mice produce mature B and T cells derived entirely from the injected ES cells (Chen et al. (1993) Proc. Natl. Acad. Sci. USA 90: 4528).

Genetic manipulation of the injected ES cells is used for introducing defined mutations and/or exogenous DNA constructs into all of the B and/or T cells of the chimeras. Chen et al. (1993), Proc. Natl. Acad. Sci. USA 90:4528-4532) generated ES cells carrying a homozygous inactivation of the Ig heavy chain locus, which, when injected into RAG blastocysts, produced chimeras which made T cells in the absence of B cells. Transfection of a rearranged murine heavy chain into the mutant ES cells results in the rescue of B cell development and the production of both B and T cells in the chimeras.

Chimeric mice which express fully human antibodies in the absence of murine Ig heavy chain or kappa light chain synthesis can be generated. Human Ig heavy and light chain

constructs are introduced into ES cells homozygous for
inactivation of both the murine Ig heavy and kappa light chain
genes. The ES cells are then injected into blastocysts
derived from RAG2 deficient mice. The resulting chimeras

5   contain B cells derived exclusively from the injected ES cells
which are incapable of expressing murine Ig heavy and kappa
light chain genes but do express human Ig genes.

Generation of ES cells Homozygous for Inactivation of the
Immunoglobulin Heavy and Kappa Light Chain Genes

10   Mice bearing inactivated Ig heavy and kappa light
chain loci were generated by targeted deletion, in ES cells,
of Ig J_H and J_K/C_K sequences, respectively according to known
procedures (Chen et al. (1993) EMBO J. 12: 821; and Chen et
al. (1993) Int. Immunol. op.cit). The two mutant strains of

15   mice were bred together to generate a strain homozygous for
inactivation of both Ig loci. This double mutant strain was
used for derivation of ES cells. The protocol used was
essentially that described by Robertson (1987, in
Teratocarcinomas and Embryonic Stem Cells: A Practical

20   Approach, p. 71-112, edited by E.J. Robertson, IRL Press).
Briefly, blastocysts were generated by natural matings of
homozygous double mutant mice. Pregnant females were
ovariectomized on day 2.5 of gestation and the "delayed"
blastocysts were flushed from the uterus on day 7 of gestation

25   and cultured on feeder cells, to help maintain their
undifferentiated state. Stem cells from the inner cell mass
of the blastocysts, identifiable by their morphology, were
picked, dissociated, and passaged on feeder cells. Cells with
a normal karyotype were identified, and male cell lines will

30   be tested for their ability to generate chimeras and
contribute to the germ cells of the mouse. Male ES cells are

preferable to female lines since a male chimera can produce
significantly more offspring.

<u>Introduction of Human Ig Genes into Mouse Ig Heavy and Kappa</u>
<u>Light Chain Deficient ES cells</u>

5   Human immunoglobulin heavy and light chain genes are
introduced into the mutant ES cells as either minilocus
constructs, such as HC2 and KC-C04, or as YAC clones, such as
J1.3P. Transfection of ES cells with human Ig DNAs is carried
out by techniques such as electroporation or lipofection with
10   a cationic lipid. In order to allow for selection of ES cells
which have incorporated the human DNA, a selectable marker
either is ligated to the constructs or is co-transfected with
the constructs into ES cells. Since the mutant ES cells
contain the neomycin phosphotransferse (neo) gene as a result
15   of the gene targeting events which generated the Ig gene
inactivations, different selectable markers, such as
hygromycin phosphotransferase (hyg) or puromycin N-acetyl
transferase (pac), are used to introduce the human Ig genes
into the ES cells.

20   The human Ig heavy and light chain genes can be
introduced simultaneously or sequentially, using different
selectable markers, into the mutant ES cells. Following
transfection, cells are selected with the appropriate
selectable marker and drug-resistant colonies are expanded for
25   freezing and for DNA analysis to verify and analyze the
integration of the human gene sequences.

<u>Generation of Chimeras</u>

              ES clones containing human Ig heavy and light chain
genes are injected into RAG-2 blastocysts as described
30   (Bradley, A. (1987), <u>in</u> Teratocarcinomas and Embryonic Stem
Cells: A Practical Approach, p. 113-151, edited by E.J.
Robertson, IRL Press) and transferred into the uteri of

pseudopregnant females. Offspring are screened for the
presence of human antibodies by ELISA assay of serum samples.
Positive animals are used for immunization and the production
of human monoclonal antibodies.

5

EXAMPLE 32

This example describes the introduction, via
homologous recombination in ES cells, of a targeted frameshift
mutation into the murine heavy chain locus leading to a
10 deletion of B cells which undergo switch recombination. The
frameshifted mice are suitable hosts for harboring non-murine
(e.g., human) transgenes encoding human sequence
immunoglobulins.

The novel frameshifted mice can be used for
15 expressing non-murine (e.g., human) sequence immunoglobulins
encoded by heavy chain transgene(s) and/or light chain
transgene(s), and for the isolation of hybridomas expressing
class-switched, affinity matured, human sequence antibodies
from introduced transgenes, among other uses. A frameshift is
20 introduced into one of the four mouse JH gene segments and
into the first exon of the mouse μ gene. The two introduced
frameshift mutations compensate for each other thus allowing
for the expression of fully functional murine μ heavy chain
when a B cell uses the frameshifted JH for a functional VDJ
25 joint. None of the other three JH segments can be used for
functional VDJ joining because of the frameshift in μ, which
is not compensated in the remaining JH genes. Alternatively,
compensating frameshifts can be engineered into multiple
murine JH genes.

30 A mouse homozygous for a compensated, frameshifted
immunoglobulin heavy chain allele has an approximately
physiological level of peripheral B cells, and an

approximately physiological level of serum IgM comprising both
murine and human μ. However, B cells recruited into germinal
centers frequently undergo a class switch to a non-μ isotype.
Such a class switch in B cells expressing the endogenous

5 murine μ chain leads to the expression of a non-compensated
frameshift mRNA, since the remaining non-μ C_H genes do not
possess a compensating frameshift. The resulting B cells do
not express a B cell receptor and are deleted. Hence, B cells
expressing a murine heavy chain are deleted once they reach

10 the stage of differentiation where isotype switching occurs.
However, B cells expressing heavy chains encoded by a non-
murine (e.g., human) transgene capable of isotype switching
and which does not contain such isotype-restrictive
frameshifts are capable of further development, including

15 isotype switching and/or affinity maturation, and the like.

Therefore, the frameshifted mouse has an impaired
secondary response with regard to murine heavy chain (μ) but a
significant secondary response with regard to transgene-
encoded heavy chains. If a heavy chain transgene that is

20 capable of undergoing class switching is introduced into this
mutant background, the non-IgM secondary response is dominated
by transgene expressing B cells. It is thus possible to
isolate affinity matured human sequence immunoglobulin
expressing hybridomas from these frameshifted mice. Moreover,

25 the frameshifted mice generally possess immunoprotective
levels of murine IgM, which may be advantageous where the
human heavy chain transgene can encode only a limited
repertoire of variable regions.

For making hybridomas secreting human sequence

30 monoclonal antibodies, transgenic mutant mice are immunized;
their spleens fused with a myeloma cell line; and the
resulting hybridomas screened for expression of the transgene

encoded human non-μ isotype. Further, the frameshifted mouse
may be advantageous over a JH deleted mouse because it will
contain a functional μ switch sequence adjacent to a
transcribed VDJ which serves as an active substrate for cis-

5   switching (Gu et al. (1993) Cell 73: 1155); thus reducing the
level of trans-switched B cells that express chimeric
human/mouse antibodies.

Construction of Frameshift Vectors

        Two separate frameshift vectors are built. One of
10 the vectors is used to introduce 2 nucleotides at the 3' end
of the mouse J4 gene segment, and one of the vectors is used
to delete those same two nucleotides from the 5' end of exon 1
of the mouse μ gene.

15 1. JH vector.

        A 3.4 kb XhoI/EcoRI fragment covering the mouse
heavy chain J region and the μ intronic enhancer is subcloned
into a plasmid vector that contains a neomycin resistance gene
as well as a herpes thymidine kinase gene under the control of
20 a phosphoglycerate kinase promoter (tk/neo cassette; Hasty et
al., (1991) Nature 350: 243). This clone is then used as a
substrate for generating 2 different PCR fragments using the
following oligonucleotide primers:

25 o-A1   5'- cca cac tct gca tgc tgc aga agc ttt tct gta -3'
    o-A2   5'- ggt gac tga ggt acc ttg acc cca gta gtc cag -3'
    o-A3   5'- ggt tac ctc agt cac cgt ctc ctc aga ggt aag aat
    ggc ctc -3'
    o-A4   5'- agg ctc cac cag acc tct cta gac agc aac tac -3'

30

        Oligonucleotides o-A1 and o-A2 are used to amplify a
1.2 kb fragment which is digested with SphI and KpnI.

Oligonucleotides o-A3 and o-A4 are used to amplify a 0.6 kb
fragment which is digested with KpnI and XbaI. These two
digested fragments are then cloned into SphI/XbaI digested
plasmid A to produce plasmid B.

5   Plasmid B contains the 2 nucleotide insertion at the
end of the J4 and, in addition, contains a new KpnI site
upstream of the insertion. The KpnI site is used as a
diagnostic marker for the insertion.

        Additional flanking sequences may be cloned into the
10   5' XhoI site and the 3' EcoRI site of plasmid B to increase
its homologous recombination efficiency. The resulting
plasmid is then digested with SphI, or another restriction
enzyme with a single site within the insert, and
electroporated into embryonic stem cells which are then
15   selected with G418 as described by Hasty et al. (1991) op.cit.
Homologous recombinants are identified by Southern blot
hybridization and then selected with FIAU as described by
Hasty et al. to obtain deleted subclones which contain only
the 2 base pair insertion and the new KpnI site in JH4. These
20   are identified by Southern blot hybridization of KpnI digested
DNA and confirmed by DNA sequence analysis of PCR amplified
JH4 DNA.

        The resulting mouse contains a JH4 segment that has
been converted from the unmutated sequence:
25   ...TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG__gtaagaatggcctctcc...
     TrpGlyGlnGlyThrSerValThrVAlSerSerGlu

to the mutant sequence:
     ...TGGGGTCAAGGTACCTCAGTCACCGTCTCCTCAGAGgtaagaatggcctctcc...
     TrpGlyGlnGlyThrSerValThrVAlSerSerGlu

30

μ Exon 1 Vector

Using similar *in vitro* mutagenesis methodology described above to engineer a two base pair insertion into the JH4 gene segment, PCR products and genomic subclones are assembled to create a vector containing a two base pair

5   deletion at the 5' end of the first μ exon. In addition, to mark the mutation, a new XmnI site is also introduced downstream by changing an A to a G.

The sequence of the unmutated μ gene is:

...ctggtcctcagAGAGTCAGTCCTTCCCAAATGTCTTCCCCCTCGTC...

10   GluSerGlnSerPheProAsnValPheProLeuVal

The sequence of the mutated μ gene is:

XmnI

...ctggtcctcag__AGTCAGTCCTTCCCGAATGTCTTCCCCCTCGTC...

SerGlnSerPheProAsnValPheProLeuVal

15   The homologous recombination vector containing the mutant sequence is linearized and electroporated into an ES cell line containing the JH4 insertion. Homologous recombinants are identified from neomycin-resistant clones. Those homologous recombinants that contain the frameshift insertion on the same

20   chromosome as the JH4 insertion are identified by Southern blot hybridization of KpnI/BamHI digested DNA. The JH4 insertion is associated with a new KpnI site that reduces the size of the J-μ intron containing KpnI/BamHI fragment from the wild type 11.3 kb to a mutant 9 kb. The resulting clones are

25   then selected for deletion of the inserted tk/neo cassette using FIAU. Clones containing the mutant μ exon are identified by Southern blot hybridization of XmnI digested DNA. The mutation is confirmed by DNA sequence analysis of PCR amplified μ exon1 DNA.

30   <u>Generation of Frameshifted Mice</u>

The ES cell line containing both the two base pair insertion in JH4, and the two base pair deletion in μ exon 1,

is then introduced into blastocyst stage embryos which are
inserted into pseudopregnant females to generate chimeras.
Chimeric animals are bred to obtain germline transmission, and
the resulting animals are bred to homozygosity to obtain
5   mutant animals homozygous for compensated frameshifted heavy
chain loci and having impaired secondary humoral immune
responses in B cells expressing murine heavy chains.

A human heavy chain transgene, such as for example
pHC1 or pHC2 and the like, may be bred into the murine heavy
10   chain frameshift background by crossbreeding mice harboring
such a human transgene into mice having the frameshifted
murine IgH locus. Via interbreeding and backcrossing, mice
homozygous at the murine IgH locus for μ-compensated
frameshifted murine IgH alleles (i.e., capable of compensated
15   in-frame expression of only murine μ and not murine non-μ
chains) and harboring at least one integrated copy of a
functional human heavy chain transgene (e.g., pHC1 or pHC2)
are produced. Such mice may optionally contain knockout of
endogenous murine κ and/or λ loci as described supra, and may
20   optionally comprise a human or other non-murine light chain
transgene (e.g., pKC1e, pKC2, and the like).

Alternatively, the human transgene(s) (heavy and/or
light) may comprise compensating frameshifts, so that the
transgene J gene(s) contain a frameshift that is compensated
25   by a frameshift in the transgene constant region gene(s).
Trans-switching to the endogenous constant region genes is
uncompensated and produces a truncated or nonsense product; B
cells expressing such uncompensated trans-switched
immunoglobulins are selected against and depleted.

30

EXAMPLE 33

Endogenous Heavy Chain Inactivation by D Region Ablation

This example describes a positive-negative selection homologous recombination vector for replacing the mouse germline immunoglobulin heavy chain D region with a nonfunctional rearranged VDJ segment. The resulting allele

5   functions within a B cell as a normal non-productive allele, with the allele undergoing intra-allele heavy chain class switching, thereby reducing the level of trans-switching to an active transgene locus.

D Region Targeting Construct

10   An 8-15 kb DNA fragment located upstream of the murine D region is isolated and subcloned from a mouse strain 129 phage library using an oligonucleotide probe comprising approximately 50 consecutive nucleotides of the published sequence for the DFL16.1 segment listed in GenBank. DFL16.1

15   is the upstream D segment (i.e., proximal to the V region gene cluster and distal to the constant region gene cluster).

             Similarly, a 9.5 kb BamHI fragment containing JH3, JH4, Eμ, Sμ, and the first two coding exons of the μ constant region is isolated and subcloned from a mouse strain 129

20   genomic phage library.

             A 5-10 kb rearranged VDJ is then isolated from a mouse hybridoma (any strain) and a synthetic linker containing a stop codon is inserted into the J segment. The stop linker within the J is preferable to an out-of-frame VDJ junction

25   because of the possibility of V replacement rearrangements.

             These three fragments are assembled together with a PGKneo positive selection cassette and a PGKHSVtk negative selection cassette to form a positive-negative selection vector for eliminating the mouse D region in 129-derived ES

30   cells (e.g., AB1) by homologous recombination. The targeting vector is formed by ligating the 8-15 kb DNA fragment to the positive selection cassette (e.g., PGKneo), which is itself

ligated to the rearranged 5-10 kb rearranged VDJ, which is itself ligated to the 9.5 kb BamHI fragment; the negative selection cassette (e.g., PGKHSVtk) is then ligated at either end of the targeting construct. The construction of such a D

5   region targeting vector is shown schematically in Fig. 63.

The D region targeting construct is transferred into AB1 ES cells, positive and negative selection is performed as described above, and correctly targeted ES cells are cloned. The correctly targeted ES cell clones are used for blastocyst

10 injections and chimeric mice are produced. The chimeric mice are bred to produce founder mice harboring a D-region inactivated heavy chain allele. Interbreeding of offspring is performed to produce homozygotes lacking a functional endogenous heavy chain locus. Such homozygotes are used to

15 crossbreed to mice harboring human Ig transgenes (e.g., pHC1, pHC2, pKC2, pKC1e, KCo4) to yield (by further backcrossing to the homozygotes lacking a functional D-region) mice lacking a functional endogenous heavy chain locus and harboring a human heavy transgene (and preferably also a human light chain

20 transgene). In embodiments where some functional endogenous light chain loci remain (e.g., λ loci), it is generally preferred that transgenes contain transcriptional control sequences that direct high level expression of human light chain (e.g., κ) polypeptides, and thus allow the transgene

25 locus to compete effectively with the remaining endogenous light chain (e.g., λ) loci. For example, the Co4 kappa light chain transgene is generally preferred as compared to pKC1 with regard to the ability to compete effeectively with the endogenous λ loci in the transgenic animal.

30

EXAMPLE 34

This example describes expansion of the human light
chain transgene V gene repertoire by co-injection of a human κ
light chain minilocus and a yeast artificial chromosome
comprising a portion of the human Vκ locus.

5   Introduction of Functional Human Light Chain V Segments by Co-
Injection of Vκ-Containing YAC DNA and a κ Minilocus

An approximately 450 kb YAC clone containing part of
the human Vκ locus was obtained as a non-amplified YAC DNA
from clone 4x17E1 of the publicly available ICRF YAC library

10   (Larin et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4123;
Genome Analysis Laboratory, Imperial Cancer Research Fund,
London, UK). The 450 kb YAC clone was isolated without prior
amplification by standard pulsed-field gel electrophoresis as
per the manufacturer's specifications (CHEF DR-II

15   electrophoresis cell, Bio-Rad Laboratories, Richmond, CA).
Six individual pulse field gels were stained with ethidium
bromide and the gel material containing the YAC clone DNA was
excised from the gel and then embedded in a new (low melting
point agarose in standard gel buffer) gel cast in a triangular

20   gel tray. The resulting triangular gel (containing the six
excised YAC-containing gel blocks) was extended at the apex
with a narrow agarose gel with 2 M NaOAc in addition to the
standard electrophoresis buffer. The gel was then placed in
an electrophoresis chamber immersed in standard gel buffer.

25   The Y-shaped gel former rises above the surface of the buffer
so that current can only flow to the narrow high salt gel
portion. A plexiglas block was placed over the high salt gel
slice to prevent diffusion of the NaOAc into the buffer. The
YAC DNA was then electrophoresed out of the original excised

30   gel sliced (embedded) and into the narrow high salt gel
portion. At the point of transition from the low salt gel to
the high salt gel, there is a resistance drop that effectively

halts the migration of the DNA at the apex of the triangular

gel.

Following electrophoresis and staining with ethidium

bromide, the concentrated YAC DNA was cut away from the rest

5 of the gel and the agarose was digested with GELase (EpiCentre

Technologies, Madison, Wisconsin). Cesium chloride was then

added to the resultant YAC-containing liquid to obtain a

density of 1.68 g/ml. This solution was centrifuged at 37,000

rpm for 36 hours to separate the YAC DNA from any

10 contaminating material. 0.5 ml fractions of the resulting

density gradient were isolated and the peak DNA fraction was

dialyzed against 5 mM Tris (pH 7.4), 5 mM NaCl, 0.1 M EDTA.

Following dialysis, the concentration of the resulting 0.65 ml

solution of YAC DNA was found to contain 2 μg/ml of DNA. This

15 YAC DNA was mixed with purified DNA insert from plasmids pKC1B

and pKV4 at a ratio of 20:1:1 (micrograms YAC4x17E1:KC1B:KV4).

The resulting 2 μg/ml solution was injected into the pronuclei

of half-day B6CBF2 embryos, and 95 surviving microinjected

embryos were transferred into the oviducts of pseudopregnant

20 females. Twelve mice which developed from the microinjected

embryos were born.

EXAMPLE 35

This example describes class-switching, somatic

25 mutation, and B cell development in immunized transgenic mice

homozygous for an inactivated endogenous immunoglobulin locus

and containing the HC1 or HC2 heavy chain transgene(s).

To demonstrate that a human sequence germline

configuration minilocus can functionally replace the authentic

30 locus, we bred a mouse strain lacking endogenous IgH with

strains containing human germline-configuration IgH

transgenes. The two transgene miniloci, HC1 and HC2, include

one and four functional variable (V) segments respectively 10
and 16 diversity (D) segments respectively, all six joining
(JH) segments, and both the μ and γ1 constant region segments.
The miniloci include human cis-acting regulatory sequences--

5   such as the JH-μ intronic enhancer and the μ and γ1 switch
sequences--that are closely linked to the coding segments.
They also include an additional enhancer element derived from
the 3' end of the rat IgH locus. We crossed HC1 and HC2
transgenic mice with stem-cell derived mutant mice that lack

10   JH segments (JHD mice) as described (supra) and cannot
therefore undergo functional heavy chain rearrangements. The
resulting transgenic-JHD mice contain B cells that are
dependent on the introduced heavy chain sequences.

15   Immunizations and hybridomas.

        We immunized mice by intraperitoneal injections of
50-100μg of antigen. Antigens included human carcinoembryonic
antigen (CEA; Crystal Chem, chicago, IL), hen eggwhite
lysozyme (HEL; Pierce, Rockford, IL), and keyhole limpet

20   hemocyanin (KLH; Pierce, Rockford, IL). For primary
injections we mixed the antigen with complete Freund's
adjuvant, for subsequent injections we used incomplete
Freund's adjuvant (Gibco BRL, Gaithersburg, MD). We fused
spleen cells with the non-secreting mouse myeloma P3X63-

25   Ag8.653 (ATCC, CRL1580). We assayed serum samples and
hybridoma supernatants for the presence of specific and non-
specific antibody comprising human heavy chain sequences by
ELISA. For detection of non-specific antibodies we coated
microtiter wells with human heavy chain isotype specific

30   antibody (mouse MAb α human IgG1, clone HP6069, Calbiochem, La
Jolla, CA; mouse MAb α human IgM, clone CH6, The Binding Site,
Birmingham, UK) and developed with peroxidase conjugated

antisera (horseradish peroxidase conjugated affinity purified
fab fragment from polyclonal goat α human IgG(fc), cat # 109-
036-098; affinity purified horseradish peroxidase conjugated
polyclonal rabbit α human IgM(fc), cat # 309-035-095. Jackson
5   Immuno Research, West Grove, PA). For detection of antigen-
specific antibodies we coated microtiter wells with antigen
and developed with peroxidase-conjugated human heavy chain
isotype specific antisera. We detected bound peroxidase by
incubation with hydrogen peroxide and
10   2,2'-Azino-bis-(3-Ethylbenzthiazoline-6-Sulfonic Acid, Sigma
Chem. Co., St. Louis, MO). The reaction product is measured
by absorption at 415 nm, and corrected for absorption at
490 nm.

15   _Flow cytometry._

        We prepared single cell suspensions from spleen,
bone marrow, and peritoneal cavity, and lysed red cells with
NH₄Cl, as described by Mishell and Shiigi. The lymphocytes are
stained with the following reagents: Phycoerythrin conjugated
20   anti-mouse Igκ (clone X36; Becton Dickinson, San Jose, CA),
FITC conjugated anti-mouse IgD (clone SBA 1, Southern Biotech,
AL), FITC conjugated anti-mouse CD5 (clone 53-7.3; Becton
Dickinson, San Jose, CA), FITC conjugated anti-mouse Igλ
(clone R26-46; Pharmingen, San Diego, CA), and Cy-Chrome
25   conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San
Diego, CA). We analyzed the stained cells using a FACScan
flow cytometer and LYSIS II software (Becton Dickinson, San
Jose, CA). Most macrophages, neutrophils, and residual red
cells are excluded by gating on forward and side scatter.
30

      Rescue of B cell compartment

In the peritoneal cavity of HC1 transgenic-JHD animals we find normal levels of CD5$^+$ B cells and approximately one-quarter the normal level of conventional CD5$^-$ B cells. The transgenic peritoneal CD5$^+$ B cells are similar to the so-called

5   B-1 cells described in normal animals: they are larger than conventional B and T lymphocytes, they express lower levels of B220 than the conventional B cells found in the spleen, and they include a higher proportion of λ light chain expressing cells. Over 90% of the splenic B cells express κ, while up to

10 50% of the peritoneal B cells express λ. Thus, while the level of conventional B cells is uniformly reduced in all tissues, the level of B-1, which are reported to have a much greater capacity for self-renewal, appears to be normal in the HC1 transgenic-JHD animals.

15

Class switching.

In transgenic-JHD mice, repeated exposure to antigen results in the production of human γ1 antibodies as well as μ antibodies. We injected human CEA into transgenic-JHD mice at

20 weekly intervals and monitored the serum levels of antigen-specific IgM and IgG1 over a period of four weeks (Fig. 63). At one week there is a detectable IgM response but no IgG1 response. However, the IgG1 response is greater than the IgM response after two weeks, and it continues to increase while

25 the IgM response remains relatively constant. This pattern-- an initial IgM reaction followed by an IgG reaction--is typical of a secondary immune response; and it suggests that cis-acting sequences included in the transgene may be responding to cytokines that direct class switching. We have

30 considered three possible mechanisms for expression of non-μ isotypes, each of which have been discussed in the literature. These mechanisms are: alternative splicing, which does not

involve deletion of the μ gene; "δ-type" switching, which
involved deletion of the μ gene via homologous recombination
between flanking repeat sequences; and non-homologous
recombination between switch regions. The results of our
5   experiments, described below, are indicative of a switch
region recombination model.

         Two types of non-deletional alternative splicing
mechanisms can be invoked to explain an isotype shift. First,
it is possible that a single transcript covering both μ and γ1
10 is expressed from the transgene; this transcript could be
alternatively spliced in response to cytokines induced by
exposure to antigen. Alternative, a cytokine induced sterile
transcript initiating upstream of γ1 could be trans-spliced to
the μ transcript. If either of these mechanisms were
15 responsible for the expression of human γ1 sequences, then we
would expect to be able to isolate hybridomas that express
both μ and γ1. However, although we have screened several
hundred hybridomas expressing either human μ or human γ1, we
have not found any such double producer (μ⁺, γ1⁺) hybridomas.
20 This indicates that expression of γ1 is accompanied by
deletion of the μ gene.

         Deletion of the μ gene can be mediated by non-
homologous recombination between the μ and γ1 switch regions,
or by homologous recombination between the two flanking 400 bp
25 direct repeats (σμ and Σμ) that are included in the HC1 and
HC2 transgenes. Deletional recombination between σμ and Σμ
has been reported to be responsible for the IgD⁺, IgM⁻
phenotype of some human B cells. While the first mechanism,
non-homologous switch recombination, should generate switch
30 products of varying lengths, the second mechanism, σμ/Σμ
recombination, should always generate the same product. We
performed a Southern blot analysis of genomic DNA isolated

from three hybridomas (Fig. 64A), one expressing μ and two
expressing $\gamma 1$. We find genomic rearrangements upstream of the
transgene $\gamma 1$ only in the two the $\gamma 1$ switch regions (Fig. 64B).
Furthermore, neither of the observed structures is compatible

5   with homologous recombination between $\sigma\mu$ and $\Sigma\mu$. Our results
are therefore consistent with a model for $\gamma 1$ isotype
expression mediated by deletional non-homologous recombination
between the transgene encoded μ and $\gamma 1$ switch regions.

10   <u>Trans-switching.</u>

In addition to human $\gamma 1$, we find mouse γ in the
serum of HC1 and HC2 transgenic-JHD mice. We have also
obtained mouse γ expressing hybridomas from these animals.
Because the non-transgenic homozygous JHD animals do not

15   express detectable levels of mouse immunoglobulins, we
attribute the expression of mouse γ in the HC1 and HC2
transgenic-JHD animals to the phenomenon of trans-switching.
All of the transgenic hybridomas that we have analyzed express
either mouse or human constant region sequences, but not both.

20   It is therefore unlikely that a trans-splicing mechanism is
involved. We used PCR amplification to isolate cDNA clones of
trans-switch products, and determined the nucleotide sequence
of 10 of the resulting clones (Fig. 65). The 5'
oligonucleotide in the PCR amplification is specific for the

25   transgene encoded VH251, and the 3' oligonucleotide is
specific for mouse $\gamma 1$, $\gamma 2b$, and $\gamma 3$ sequences. We find
examples of trans-switch products incorporating all three of
these mouse constant regions.

30   <u>Somatic mutation</u>.

Approximately 1% of the nucleotides within the
variable regions of the trans-switch products shown in Fig. 7

are not germline encoded. This is presumably due to somatic
mutation. Because the mutated sequence has been translocated
to the endogenous locus, the cis-acting sequences directing
these mutations could be located anywhere 3' of the mouse γ

5   switch. However, as we discuss below, we also observe somatic
mutation in VDJ segments that have not undergone such
translocations; and this result indicates that sequences
required by heavy chain somatic mutation are included in the
transgene.

10   To determine if the HC1 and HC2 constructs include
sufficient cis-acting sequences for somatic mutation to occur
in the transgenic-JHD mice, we isolated and partially
sequenced cDNA clones derived from two independent HC1
transgenic lines and one HC2 line. We find that some of the

15   γ1 transcripts from transgenic-JHD mice contain V regions with
extensive somatic mutations. The frequency of these mutated
transcripts appears to increase with repeated immunizations.
Figs. 66A and 66B show two sets of cDNA sequences: one set is
derived form an HC1 (line 26) transgenic-JHD mouse that we

20   immunized with a single injection of antigen 5 days before we
isolated RNA; the second set is derived from an HC1 (line 26)
transgenic-JHD mouse that we hyperimmunized by injecting
antigen on three different days beginning 5 months before we
isolated RNA; the second set is derived from an HC1 (line 26)

25   transgenic-JHD mouse that we hyperimmunized by injecting
antigen on three different days beginning 5 months before we
isolated RNA. Only 2 of the 13 V regions from the 5 day post-
exposure mouse contain any non-germline encoded nucleotides.
Each of these V's contains only a single nucleotide change,

30   giving an overall somatic mutation frequency of less than 0.1%
for this sample. In contrast, none of the 13 V sequences from

the hyperimmunized animal are completely germline, and the
overall somatic mutation frequency is 1.6%.

Comparison of μ and γ1 transcripts isolated from a
single tissue sample shows that the frequency of somatic
5   mutations is higher in transgene copies that have undergone a
class switch. We isolated and partially sequenced 47
independent μ and γ1 cDNA clones from a hyperimmunized CH1
line 57 transgenic-JHD mouse (Fig. 67A and 67B). Most of the
μ cDNA clones are unmodified relative to the germline
10 sequence, while over half of the γ1 clones contain multiple
non-germline encoded nucleotides. The γ1 expressing cells are
distinct from the μ expressing cells and, while the two
processes are not necessarily linked, class switching and
somatic mutation are taking place in the same sub-population
15 of B cells.

Although we do not find extensive somatic mutation
of the VH251 gene in non-hyperimmunized CH1 transgenic mice,
we have found considerable somatic mutation in VH56p1 and
VH51p1 genes in a naive HC2 transgenic mouse. We isolated
20 spleen and lymph node RNA from an unimmunized 9 week old
female HC2 transgenic animal. We individually amplified γ1
transcripts that incorporate each of the four V regions in the
HC2 transgene using V and γ1 specific primers. The relative
yields of each of the specific PCR products were
25 VH56p1>>VH51p1>VH4.21>VH251. Although this technique is not
strictly quantitative, it may indicate a bias in V segment
usage in the HC2 mouse. Fig. 68 shows 23 randomly picked γ1
cDNA sequences derived from PCR amplifications using an
equimolar mix of all four V specific primers. Again we
30 observe a bias toward VH56p1 (19/23 clones). In addition, the
VH56p1 sequences show considerable somatic mutation, with an
overall frequency of 2.1% within the V gene segment.

Inspection of the CDR3 sequences reveals that although 17 of
the 19 individual VH56p1 clones are unique, they are derived
from only 7 different VDJ recombination events. It thus
appears that the VH56p1 expressing B cells are selected,
5   perhaps by an endogenous pathogen or self antigen, in the
naive animal. It may be relevant that this same gene is over-
represented in the human fetal repertoire.

<u>Summary</u>

10   Upstream cis-acting sequences define the
functionality of the individual switch regions, and are
necessary for class switching. Our observation--that class
switching within the HC1 transgene is largely confined to
cells involved in secondary response, and does not occur
15   randomly across the entire B cell population--suggests that
the minimal sequences contained with the transgene are
sufficient. Because the γ sequences included in this
construct begin only 116 nucleotides upstream of the start
site of the γ1 sterile transcript, the switch regulatory
20   region is compact.

             Our results demonstrate that these important cis-
acting regulatory elements are either closely linked to
individual γ genes, or associated with the 3' heavy chain
enhancer included in the HC1 and HC2 transgenes. Because the
25   HC1 and HC2 inserts undergo transgene-autonomous class
switching--which can serve as a marker for sequences that are
likely to have been somatically mutated--we were able to
easily find hypermutated transcripts that did not originate
from translocations to the endogenous locus. We found
30   somatically mutated γ transcripts in three independent
transgenic lines (two HC1 lines and one HC2 line). It is
therefore unlikely that sequences flanking the integration

sites of the transgene affect this process; instead, the transgene sequences are sufficient to direct somatic mutation.

EXAMPLE 36

5   This example describes the generation of hybridomas from mice homozygous for an inactivated endogenous immunoglobulin locus and containing transgene sequences encoding a human sequence heavy chain and human sequence light chain. The hybridomas described secrete monoclonal antibodies

10   comprising a human sequence heavy chain and a human seqeunce light chain and bind to a predetermined antigen expressed on T lymphocytes. The example also demonstrates the capacity of the mice to make a human sequence antibody in response to a human-derived immunogen, human CD4, and the suitability of

15   such mice as a source for making hybridomas secreting human sequence monoclonal antibodies reactive with human antigens.

A.   **Generation of Human Ig Monoclonal Antibodies Derived from HC1 Transgenic Mice Immunized with a Human CD4 Antigen**

20   A transgenic mouse homozygous for a functionally disrupted J_H locus and harboring a transgene capable of rearranging to encode a human sequence heavy chain and a transgene capable of rearranging to encode a human sequence light chain was immunized. The genotype of the mouse was HC1-

25   26$^+$ KC1e-1536$^+$ $J_HD^{+/+}$ J_KD^-, indicating homozygosity for murine heavy chain inactivation and the presence of germline copies of the HC1 human sequence heavy chain transgene and the KC1e human sequence light chain transgene.

            The mouse was immunized with a variant of the EL4

30   cell line (ATCC) expressing a mouse-human hybrid CD4 molecule encoded by a stably transfected polynucleotide. The expressed CD4 molecule comprises a substantially human-like CD4

sequence. Approximately 5×10^6 cells in 100 μl of PBS accompanied by 100 μl of Complete Freund's Adjuvant (CFA) were introduced into the mouse via intraperitoneal injection on Day 0. The inoculation was repeated on Days 7, 14, 21, 28, 60, and 77, with test bleeds on Days 18, 35, and 67. The spleen was removed on Day 81 and approximately 7.2×10^7 spleen cells were fused to approximately 1.2×10^7 fusion partner cells (P3x63Ag8.653 cell line; ATCC) by standard methods (PEG fusion) and cultured in RPMI 1640 15 % FCS, 4 mM glutamine, 1 mM sodium pyruvate plus HAT and PSN medium. Multiple fusions were performed.

Hybridomas were grown up and supernatants were tested with ELISA for binding to a commercial source of purified recombinant soluble human sequence CD4 expressed in CHO cells (American Bio-Technologies, Inc. (ABT), Cambridge, MA) and/or CD4 obtained from NEN-DuPont. The ABT sample contained a purified 55 kD human CD4 molecule comprised the V_1 through V_3 domains of human CD4. The recombinant human sequence CD4 (produced in CHO-K1 cells) was adsorbed to the assay plate and used to capture antibody from hybridoma supernatants, the captured antibodies were then evaluated for binding to a panel of antibodies which bind either human μ, human κ, human γ, murine μ, or murine κ.

One hybridoma was subcloned from its culture plate well, designated 1F2. The 1F2 antibody bound to the ABT CD4 preparation, was positive for human μ and human κ, and was negative for human γ, mouse γ, and mouse κ.

B.   <u>Generation of Human Ig Monclonal Antibodies Derived from HC2 Transgenic Mice Immunized with Human CD4 and Human IgE.</u>

The heavy chain transgene, HC2, is shown in Fig. 56 and has been described <u>supra</u> (see, Example 34).

The human light chain transgene, KCo4, depicted in
Fig. 56 is generated by the cointegration of two individually
cloned DNA fragments at a single site in the mouse genome.
The fragments comprise 4 functional Vκ segments, 5J segments,
5   the Cκ exon, and both the intronic and downstream enhancer
elements (see Example 21) (Meyer and Neuberger (1989), EMBO J.
8:1959-1964; Judde and Max (1992), Mol. Cell Biol. 12:5206-
5216). Because the two fragments share a common 3 kb sequence
(see Fig. 56), they can potentially integrate into genomic DNA
10   as a contiguous 43 kb transgene, following homologous
recombination between the overlapping sequences. It has been
demonstrated that such recombination events frequently occur
upon microinjection of overlapping DNA fragments (Pieper et
al. (1992), Nucleic Acids Res. 20:1259-1264). Co-injected
15   DNA's also tend to co-integrate in the zygote, and the
sequences contained within the individually cloned fragments
would subsequently be jointed by DNA rearrangement during B
cell development. Table 12 shows that transgene inserts from
at least 2 of the transgenic lines are functional. Examples
20   of VJ junctions incorporating each of the 4 transgene encoded
V segments, and each of the 5J segments, are represented in
this set of 36 clones.

Table 12

Twenty-three light chain minilocus positive and 18 heavy chain positive mice developed from the injected embryos. These mice, and their progeny, were bred with mice containing targeted mutations in the endogenous mouse heavy (strain JHD) and κ light chain loci (strain JCKD) to obtain mice containing human heavy and κ light chain in the absence of functional mouse heavy and κ light chain loci. In these mice, the only mouse light chain contribution, if any, is from the mouse λ locus.

Table 13 show that somatic mutation occurs in the variable regions of the transgene-encoded human heavy chain transcripts of the transgenic mice. Twenty-three cDNA clones from a HC2 transgenic mouse were partially sequenced to determine the frequency of non-germline encoded nucleotides within the variable region. The data include only the sequence of V segment codons 17-94 from each clone, and does not include N regions. RNA was isolated from the spleen and lymph node of mouse 5250 (HC2 line 2550 hemizygous, JHD homozygous). Single-stranded cDNA was synthesized and γ transcripts amplified by PCR as described [references]. The amplified cDNA was cloned into plasmid vectors, and 23 randomly picked clones were partially sequenced by the dideoxy chain-termination method. The frequency of PCR-introduced nucleotide changes is estimated from constant region sequence as <0.2%.

TABLE 13: The Variable Regions of Human γ Transcripts in HC2
    Transgenic Mice Contain Non-Germline-Encoded Nucleotides

VH Segment	Number of clones	Number of non-germline encoded nucleotides	Frequency of non-germline-encoded nucleotides (%)
VH251	0		--

256

VH56P1	10	100	2.1
VH51P1	1	5	2.0
VH4.21	3	0	0.0

Flow cytometry

We analyzed the stained cells using a FACScan
flow cytometer and LYSIS II software (Becton Dickinson, San
Jose, CA). Spleen cells were stained with the following
reagents: propidium iodide (Molecular Probes, Eugene, OR),
phycoerythrin conjugated α-human Igκ (clone HP6062; Caltag, S.
San Francisco, CA), phycoerythrin conjugated α-mouse Igκ (clone
X36; Becton Dickinson, San Jose, CA), FITC conjugated α-mouse
Igλ (clone R26-46; Pharmingen, San diego, CA), FITC conjugated
α-mouse Igμ (clone R6-60.2; Pharmingen, San Diego, CA), FITC
conjugated α-human Igμ (clone G20-127; Pharmingen, San Diego,
CA), and Cy-Chrome conjugated α-mouse B220 (clone RA3-6B2;
Pharmingen, San Diego, CA).

Expression of human Ig transgenes

Figure 69 shows a flow cytometric analysis of spleen cells from
KCo4 and HC2 mice that are homozygous for both the JHD and JCKD
mutations. The human sequence HC2 transgene rescued B cell
development in the JHD mutant background, restoring the
relative number of B220⁺ cells in the spleen to approximately
half that of a wild type animal. These B cells expressed cell
surface immunoglobulin receptors that used transgene encoded
heavy chain. The human KCo4 transgene was also functional, and
competed successfully with the intact endogenous λ light chain
locus. Nearly 95% of the splenic B cells in JHD/JCKD
homozygous mutant mice that contain both heavy and light chain
human transgenes (double transgenic) expressed completely human
cell surface IgMκ.

Serum Ig levels were determined by ELISA done as
follows: human μ: microtiter wells coated with mouse Mab α
human IgM (clone CH6, The Binding Site, Birmingham, UK) and
developed with peroxidase conjugated rabbit α human IgM(fc)

5   (cat # 309-035-095, Jackson Immuno Research, West Grove, PA).
Human γ: microtiter wells coated with mouse MAb α human IgG1
(clone HP6069, Calbiochem, La Jolla, CA) and developed with
peroxidase conjugated goat α human IgG(fc) (cat # 109-036-098,
Jackson Immuno Research, West Grove, PA).   Human κ: microtiter

10   wells coated with mouse Mab α human Igκ (cat # 0173, AMAC, Inc.
Igκ (cat #A7164, Sigma Chem. Co., St. Louis, MO).   Mouse γ:
microtiter wells coated with goat α mouse IgG (cat #115-006-
071, Jackson Immuno Research, West Grove, PA).   Mouse λ:
microtiter wells coated with rat MAb α mouse Igλ (cat # 02171D,

15   Pharmingen, San Diego, CA) and developed with peroxidase
conjugated rabbit α mouse IgM(fc) (cat # 309-035-095, Jackson
Immuno Research, West Grove, PA).   Bound peroxidase is detected
by incubation with hydrogen peroxide and 2,2'-Azino-bis-)3-
Ethylbenzthiazoline-6-Sulfonic Acid, Sigma Chem. Co., St.

20   Louis, MO).   The reaction product is measured by absorption at
415 nm.

The double transgenic mice also express fully
human antibodies in the serum.   Figure 70 shows measured serum
levels of immunoglobulin proteins for 18 individual double

25   transgenic mice, homozygous for endogenous heavy and kappa
light chain inactivations, derived from several different
transgenic founder animals.   We found detectable levels of
human μ, γ1, and κ.   We have shown supra that the expressed
human γ1 results from authentic class switching by genomic

30   recombination between the transgene μ and γ1 switch regions.
Furthermore, we have found that intra-transgene class switching
was accompanied by somatic mutation of the heavy chain variable

regions. In addition to human immunoglobulins, we also found mouse γ and λ in the serum. The present of mouse λ protein is expected because the endogenous locus is completely intact. We have shown elsewhere that the mouse γ expression is a

5   consequence of trans-switch recombination of transgene VDJ segments into the endogenous heavy chain locus. This trans-switching phenomenon, which was originally demonstrated for wild-type heavy chain alleles and rearranged VDJ transgenes (Durdik et al. (1989), <u>Proc. Natl. Acad. Sci. USA</u> 86:2346-2350;

10   Gerstein et al. (1990), <u>Cell</u> 63:537-548), occurs in the mutant JHD background because the downstream heavy chain constant regions and their respective switch elements are still intact.

The serum concentration of human IgMκ in the double transgenic mice was approximately 0.1 mg/ml, with very

15   little deviation between animals or between lines. However, human γ1, mouse γ, and mouse λ levels range from 0.1 to 10 micrograms/ml. The observed variation in γ levels between individual animals may be a consequence of the fact that γ is an inducible constant region. Expression presumably depends on

20   factors such as the health of the animal, exposure to antigens, and possibly MHC type. The mouse λ serum levels are the only parameter that appears to correlate with individual transgenic lines. KCo4 line 4436 mice which have the fewest number of copies of the transgene per integration (approximately 1-2

25   copies) have the highest endogenous λ levels, while KCo4 line 4437 mice (~10 copies per integration) have the lowest λ levels. This is consistent with a model in which endogenous λ rearranges subsequent to the κ transgene, and in which the serum λ level is not selected for, but is instead a reflection

30   of the relative size of the precursor B cell pool. Transgene loci containing multiple light chain inserts may have the opportunity to undergo more than one V to J recombination

event, with an increased probability that one of them will be functional. Thus high copy lines will have a smaller pool of potential λ cells.

5   Immunizations with human CD4 and IgE

To test the ability of the transgenic B cells to participate in an immune response, we immunized double transgenic mice with human protein antigens, and measured serum levels of antigen specific immunoglobulins by ELISA. Mice were

10   immunized with 50 μg recombinant sCD4 (cat. # 013101, American Bio-Technologies Inc., Cambridge, MA) covalently linked to polystyrene beads (cat # 08226, Polysciences Inc., Warrington, PA) in complete Freund's adjuvant by intraperitoneal injection. Each of the mice are homozygous for disruptions of the

15   endogenous μ and κ loci, and hemizygous for the human heavy chain transgene HC2 line 2500 and human κ light chain transgene KCo4 line 4437.

Methods

Serum samples were diluted into microtiter wells

20   coated with recombinant sCD4. Human antibodies were detected with peroxidase conjugated rabbit α human IgM(fc) (Jackson Immuno Research, West Grove, PA) or peroxidase conjugated goat anti-human Igκ (Sigma, St. Louis, MO).

Figure 71A shows the primary response of

25   transgenic mice immunized with recombinant human soluble CD4. All four of the immunized animals show an antigen-specific human IgM response at one week. The CD4-specific serum antibodies comprise both human μ heavy chain and human κ light chain.

30   To evaluate the ability of the HC2 transgene to participate in a secondary response, we hyperimmunized the transgenic mice by repeated injection with antigen, and

monitored the heavy chain isotype of the induced antibodies. Mice homozygous for the human heavy chain transgene HC2 and human κ light chain transgene KCo4 were immunized with 25 μg of human IgEκ (The Binding Site, Birmingham, UK) in complete

5 Freund's adjuvant on day = 0. Thereafter, animals were injected with IgEκ in incomplete Freund's adjuvant at approximately weekly intervals. Serum samples were diluted 1:10, and antigen-specific ELISAs were performed on human IgE, λ coated plates.

10 Figure 71B shows a typical time course of the immune response from these animals: we injected double transgenic mice with human IgE in complete Freund's adjuvant, followed by weekly boosts of IgE in incomplete Freund's adjuvant. The initial human antibody response was IgMκ,

15 followed by the appearance of antigen specific human IgGκ. The induced serum antibodies in these mice showed no cross-reactivity to human IgM or BSA. The development, over time, of a human IgG

We have also tested the ability of the heavy

20 chain transgene to undergo class switching *in vitro*: splenic B cells purified form animals hemizygous for the same heavy chain construct (HC2, line 2550) switch from human IgM to human IgG1 in the presence of LPS and recombinant mouse IL-4. However, *in vitro* switching did not take place in the presence of LPS and

25 recombinant mouse IL-2, or LPS alone.

We find human IgM-expressing cells in the spleen, lymph nodes, peritoneum, and bone marrow of the double-transgenic/double-knockout (0011) mice. Although the peritoneal cavity contains the normal number of B cells, the

30 absolute number of transgenic B cells in the bone marrow and spleen is approximately 10-50% of normal. The reduction may result from a retardation in transgene-dependent B cell

development. The double-transgenic/double-knockout (0011) mice also express fully human antibodies in the serum, with significant levels of human μ, $\gamma 1$, and κ in these mice. The expressed human $\gamma 1$ results from authentic class switching by genomic recombination between the transgene μ and $\gamma 1$ switch regions. Furthermore, the intratransgene class switching is accompanied by somatic mutation of the heavy chain variable regions encoded by the transgene. In addition to human immunoglobulins, we find mouse μ and mouse λ in these mice. The mouse μ expression appears to be a result of trans-switching recombination, wherein transgene VDJ gene is recombined into the endogenous mouse heavy chain locus. Trans-switching, which was originally observed in the literature for wild-type heavy chain alleles and rearranged VDJ transgenes, occurs in our $J_H^{-/-}$ background because the mouse downstream heavy chain constant regions and their respective switch elements are still intact.

To demonstrate the ability of the transgenic B cells to participate in an immune response, we immunized the 0011 mice with human protein antigens, and monitored serum levels of antigen-specific immunoglobulins. The initial human antibody response is IgM, followed by the expression of antigen-specific human IgG (Fig. 71B and Fig. 73). The lag before appearance of human IgG antibodies is consistent with an association between class-switching and a secondary response to antigen.

In a transgenic mouse immunized with human CD4, human IgG reactivity to the CD4 antigen was detectable at serum concentrations ranging from 2×10^{-2} to 1.6×10^{-4}.

Identification of Anti-Human CD4 Hybridomas

A transgenic mouse homozygous for the human heavy chain transgene HC2 and human κ light chain transgene KCo4 were immunized with 20 μg of recombinant human CD4 in complete Freund's adjuvant on day 0. Thereafter, animals were injected

5   with CD4 in incomplete Freund's adjuvant at approximately weekly intervals. Fig. 73 shows human antibody response to human CD4 in serum of the transgenic mouse. Serum samples were diluted 1:50, and antigen-specific ELISAs were performed on human CD4 coated plates. Each line represents individual

10   sample determinations. Solid circles represent IgM, open squares represent IgG.

We also isolated hybridoma cell lines from one of the mice that responded to human CD4 immunization. Five of the cloned hybridomas secrete human IgGκ (human γ1/human κ)

15   antibodies that bind to recombinant human CD4 and do not crossreact (as measured by ELISA) with a panel of other glycoprotein antigens. The association and dissociation rates of the immunizing human CD4 antigen for the monoclonal antibodies secreted by two of the IgGκ hybridomas, 4E4.2 and

20   2C5.1, were determined. The experimentally-derived binding constants (K_a) were approximately 9×10^7 M^{-1} and 8×10^7 M^{-1} for antibodies 4E4.2 and 2C5.1, respectively. These K_a values fall within the range of murine IgG anti-human CD4 antibodies that have been used in clinical trials by others (Chen et al. (1993)

25   <u>Int. Immunol.</u> <u>6</u>: 647).

A mouse of line #7494 (0012;HC1-26+;JHD++;JKD++;KC2-1610++) was immunized on days 0, 13, 20, 28, 33, and 47 with human CD4, and produced anti-human CD4 antibodies comprised of human κ and human μ or γ.

30   By day 28, human μ and human κ were found present in the serum. By day 47, the serum response against human CD4 comprised both human μ and human γ, as well as human κ. On day

50, splenocytes were fused with P3X63-Ag8.653 mouse myeloma cells and cultured. Forty-four out of 700 wells (6.3%) contained human γ and/or κ anti-human CD4 monoclonal antibodies. Three of these wells were confirmed to contain

5 human γ anti-CD4 monoclonal antibodies, but lacked human κ chains (presumably expressing mouse λ). Nine of the primary wells contained fully human IgMκ anti-CD4 monoclonal antibodies, and were selected for further characterization. One such hybridoma expressing fully human IgMκ anti-CD4

10 monoclonal antibodies was designated 2C11-8.

Primary hybridomas were cloned by limiting dilution and assessed for secretion of human μ and κ monoclonal antibodies reactive against CD4. Five of the nine hybridomas remained positive in the CD4 ELISA. The specificity of these

15 human IgMκ monoclonal antibodies for human CD4 was demonstrated by their lack of reactivity with other antigens including ovalbumin, bovine serum albumin, human serum albumin, keyhole limpet hemacyanin, and carcinoembryonic antigen. To determine whether these monoclonal antibodies could recognize CD4 on the

20 surface of cells (i.e., native CD4), supernatants from these five clones were also tested for reactivity with a CD4+ T cell line, Sup T1. Four of the five human IgMκ monoclonal antibodies reacted with these CD4+ cells. To further confirm the specificity of these IgMκ monoclonal antibodies, freshly

25 isolated human peripheral blood lymphocytes (PBL) were stained with these antibodies. Supernatants from clones derived from four of the five primary hybrids bound only to CD4+ lymphocytes and not to CD8+ lymphocytes (Figure 72).

Fig. 72 shows reactivity of IgMκ anti-CD4

30 monoclonal antibody with human PBL. Human PBL were incubated with supernatant from each clone or with an isotype matched negative control monoclonal antibody, followed by either a

mouse anti-human CD4 monoclonal antibody conjugated to PE (top row) or a mouse anti-human CD8 Ab conjugated to FITC (bottom row). Any bound human IgMĸ was detected with a mouse anti-human μ conjugated to FITC or to PE, respectively.

5   Representative results for one of the clones, 2C11-8 (right side) and for the control IgMĸ (left side) are shown. As expected, the negative control IgMĸ did not react with T cells and the goat anti-human μ reacted with approximately 10% of PBL, which were presumably human B cells.

10   Good growth and high levels of IgMĸ anti-CD4 monoclonal antibody production are important factors in choosing a clonal hybridoma cell line for development. Data from one of the hybridomas, 2C11-8, shows that up to 5 pg/cell/d can be produced (Figure 74). Similar results were

15   seen with a second clone. As is commonly observed, production increases dramatically as cells enter stationary phase growth. Fig. 74 shows cell growth and human IgMĸ anti-CD4 monoclonal antibody secretion in small scale cultures. Replicate cultures were seeded at $2x10^5$ cells/ml in a total volume of 2 ml. Every

20   twenty-four hours thereafter for four days, cultures were harvested. Cell growth was determined by counting viable cells and IgMĸ production was quantitated by an ELISA for total human μ (top panel). The production per cell per day was calculated by dividing the amount of IgMĸ by the cell number (bottom

25   panel).

                        Fig. 75 shows epitope mapping of a human IgMĸ anti-CD4 monoclonal antibody. Competition binding flow cytometric experiments were used to localize the epitope recognized by the IgMĸ anti-CD4 monoclonal antibody, 2C11-8.

30   For these studies, the mouse anti-CD4 monoclonal antibodies, Leu3a and RPA-T4, which bind to unique, nonoverlapping epitopes on CD4 were used. PE fluorescence of CD4+ cells preincubated

with decreasing concentrations of either RPA-TA or Leu-3a
followed by staining with 2C11-8 detected with PE-conjugated
goat anti-human IgM. 1There was concentration-dependent
competition for the binding of the human IgMκ anti-CD4

5   monoclonal antibody 2C11-8 by Leu3a but not by RPA-T4 (Figure
75). Thus, the epitope recognized by 2C11-8 was similar to or
identical with that recognized by monoclonal antibody Leu3a,
but distinct from that recognized by RPA-T4.

In summary, we have produced several hybridoma

10   clones that secrete human IgMκ monoclonal antibodies that
specifically react with native human CD4 and can be used to
discriminate human PBLs into $CD4^+$ and $CD4^-$ subpopulations. At
least one of these antibodies binds at or near the epitope
defined by monoclonal antibody Leu3a. Monoclonal antibodies

15   directed to this epitope have been shown to inhibit a mixed
leukocyte response (Engleman et al., J. Exp. Med. (1981)
153:193). A chimeric version of monoclonal antibody Leu3a has
shown some clinical efficacy in patients with mycosis fungoides
(Knox et al. (1991) Blood 77:20).

20   We have isolated cDNA clones from 3 different
hybridoma cell lines (2C11.8, 2C5.1, and 4E4.2), and have
determined the partial nucleotide sequence of some of the
expressed inununoglobuhn genes in each of these cell lines.
For sequence analysis, total RNA was isolated from

25   approximately 5×10^6 hybridoma cells. sscDNA was synthesized
by priming reverse transcription with oligo dT. A portion of
this sscDNA was used in duplicate PCR reactions primed by a
pool of oligos with specificities for either (i) heavy chain
variable framework regions contained within the HC1 or HC2

30   transgenes and a single downstream oligo specific for constant
human gamma sequence, or (ii) light chain variable framework
regions contained within the KC2 or KCo4 transgene and a single

downstream oligo specific for constant human kappa sequence.
Products from these PCR reactions were digested with
appropriate restriction enzymes, gel purified, and
independently cloned into pNNO3 vector. DNA was isolated and
5   manual dideoxy and/or automated fluorescent sequencing
reactions performed on dsDNA.

The characteristics of the three hybridomas,
2C11.8, 2C5.1, and 4E4.2, are given below in Table 11.

10   <u>Table 11 Human variable region usage in hybridomas</u>

Subclone	Specificity	Isotype	Vh	Dh	Jh	Vκ	Jκ
2C11.8	nCD4	IgMκ	251	nd.*	nd.	nd.	nd.
2C5.1	rCD4	IgGκ	251	HQ52	JH5	65.15	JK4
4E4.2	rCD4	IgGκ	251	HQ52	JH5	65.15	JK4

* n.d., not determined

Nucleotide sequence analysis of expressed heavy and
20   light chain sequences from the two IgGκ hybridomas 2C5.1 and
4E4.2 reveal that they are sibling clones derived from the same
progenitor B cell. The heavy and light chain V(D)J junctions
from the two clones are identical, although the precise
nucleotide sequences differ by presumptive somatic mutations.
25   The heavy chain VDJ junction sequence is:

```
    VH251                    N          DHQ52             JH5
TAT TAC TGT GCG AG   (g gct cc)   A ACT GGG GA   C TGG TTC GAC
 Y   Y   C   A   R      A   P        T   G   D      W   F   D
```

30

The light chain VJ junction is:

```
    Vk65.15                    N            Jk4
```

```
TAT AAT AGT TAC CCT CC        (t)        ACT TTC GGC
 Y   N   S   Y   P   P                    T   F   G
```

The following non-germline encoded codons were identified
(presumptive somatic mutations):

2CS.1 heavy chain AGC->AGG S28R   (replacement)
       light chain CCG->ACG P119T (replacement)

4E4.2 heavy chain AGC->AGG S28R (replacement)
                    CTG->CTA L80L (silent)
       light chain GAG->GAC E41D (replacement)
                    AGG->AAG R61K (replacement)
                    CCG->ACG P119T (replacement)

        We conclude that these two gamma hybridomas are
derived from B cells that have undergone a limited amount of
somatic mutation. This data shows that the HC2 transgenic
animals use the VH5-51 (aka VH251) V segment. We have
previously shown that VH4-34, VH1-69, and VH3-30.3 are
expressed by these mice. The combination of these results
demonstrates that the HC2 transgenic @ce express all four of
the transgene encoded human VH genes.

        We conclude that human immunoglobulin-expressing B
cells undergo development and respond to antigen in the context
of a mouse immune system. Antigen responsivity leads to
immunoglobulin heavy chain isotype switching and variable
region somatic mutation. We have also demonstrated that
conventional hybridoma technology can be used to obtain
monoclonal human sequence antibodies from these mice.
Therefore, these transgneic mice represent a source of human
antibodies against human target antigens.

EXAMPLE 37

This example describes the generation of transgenic
mice homozygous for an inactivated endogenous heavy chain and κ
chain locus and harboring a transgene capable of isotype
5   switching to multiple downstream human C_H genes. The example
also demonstrates a cloning strategy for assembling large
transgenes (e.g., 160 kb) by co-microinjection of multiple DNA
fragments comprising overlapping homologous sequence joints
(see Fig. 76), permitting construction of a large transgene
10   from more than two overlapping fragments by homologous
recombination of a plurality of homology regions at distal ends
of the set of fragments to be assembled in vivo, such as in a
microinjected ES cell or its clonal progeny. The example also
shows, among other things, that isolated lymphocytes from the
15   transgenic animals can be induced to undergo isotype switching
in vitro, such as with IL-4 and LPS.

A set of five different plasmid clones was
constructed such that the plasmid inserts could be isolated,
substantially free of vector sequences; and such that the
20   inserts together form a single imbricate set of overlapping
sequence spanning approximately 150 kb in length. This set
includes human V, D, J, μ, γ3, and γ1 coding sequences, as well
as a mouse heavy chain 3' enhancer sequence. The five clones
are, in 5' to 3' order: pH3V4D, pCOR1xa, p11-14, pP1-570, and
25   pHP-3a (Fig. 76). Several different cloning vectors were used
to generate this set of clones. Some of the vectors were
designed specifically for the purpose of building large
transgenes. These vectors (pGP1a, pGP1b, pGP1c, pGP1d, pGP1f,
pGP2a, and pGP2b) are pBR322-based plasmids that are maintained
30   at a lower copy number per cell than the pUC vectors
(Yanisch-Perron et al. (1985) Gene 33: 103-119). The vectors
also include trpA transcription termination signals between the

polylinker and the 3' end of the plasmid β-lactamase gene. The
polylinkers are flanked by restriction sites for the
rare-cutting enzyme NotI; thus allowing for the isolation of
the insert away from vector sequences prior to embryo

5   microinjection. Inside of the NotI sites, the polylinkers
include unique XhoI and SalI sites at either end. The pGP1
vectors are described in Taylor et al. (1992) Nucleic Acids
Res. 23: 6287. To generate the pGP2 vectors, pGP1f was first
digested with AlwNI and ligated with the synthetic

10   oligonucleotides o-236 and o-237 (o-236, 5'- ggc gcg cct tgg
cct aag agg cca -3'; o-237, 5'- cct ctt agg cca agg cgc gcc tgg
-3') The resulting plasmid is called pGP2a. Plasmid pGP2a was
then digested with KpnI and EcoRI, and ligated with the
oligonucleotides o-288 and o-289 (o-288, 5'- aat tca gta tcg

15   atg tgg tac -3'; o-289, 5'- cac atc gat act g -3') to create
pGP2b (Figs. 77A and Fig. 77B).

        The general scheme for transgene construction with
the pGP plasmids is outlined in Fig. 78 (paths A and B). All
of the component DNA fragments are first cloned individually in

20   the same 5' to 3' orientation in pGP vectors. Insert NotI,
XhoI and SalI sites are destroyed by oligonucleotide
mutagenesis or if possible by partial digestion, polymerase
fill-in, and blunt end ligation. This leaves only the
polylinker derived XhoI and SalI sites at the 5' and 3' ends of

25   each insert. Individual inserts can then be combined stepwise
by the process of isolating XhoI/SalI fragments from one clone
and inserting the isolated fragment into either the 5' XhoI or
3' SalI site of another clone (Fig. 78, path A). Transformants
are then screened by filter hybridization with one or more

30   insert fragments to obtain the assembled clone. Because
XhoI/SalI joints cannot be cleaved with either enzyme, the
resulting product maintains unique 5' XhoI and 3' SalI sites,

270

and can be used in the step of the construction. A variation
of this scheme is carried out using the vectors pGP2a and pGP2b
(Fig. 78, path B). These plasmids includes an SfiI site
between the ampicillin resistance gene and the plasmid origin

5   of replication. By cutting with SfiI and XhoI or SalI, inserts
can be isolated together with either the drug resistance
sequence or the origin of replication. One SfiI/XhoI fragment
is ligated to one SfiI/SalI fragment in each step of the
synthesis. There are three advantages to this scheme: (i)

10 background transformants are reduced because sequences from
both fragments are required for plasmid replication in the
presence of ampicillin; (ii) the ligation can only occur in a
single 5' to 3' orientation; and (iii) the SfiI ends are not
self-compatible, and are not compatible with SalI or XhoI, thus

15 reducing the level of non-productive ligation. The
disadvantage of this scheme is that insert SfiI sites must be
removed as well as NotI, XhoI, and SalI sites. These medium
copy vectors are an improvement over the commonly used pUC
derived cloning vectors. To compare the ability of these

20 vectors to maintain large DNA inserts, a 43 kb XhoI fragment
comprising the human JH/Cμ region was ligated into the SalI
site of pSP72 (Promega, Madison, WI), pUC19 (BRL, Grand Island,
NY), and pGP1f. Transformant colonies were transferred to
nitrocellulose and insert containing clones were selected by

25 hybridization with radiolabeled probe. Positive clones were
grown overnight in 3 ml media and DNA isolated: EcoRI digestion
of the resulting DNA reveals that all the pSP72 and pUC19
derived clones deleted the insert (Fig. 79); however, 12 of the
18 pGP1f derived clones contained intact inserts. Both

30 orientations are represented in these 12 clones.

The construction and isolation of the five clones
(pH3V4D, pCOR1xa, p11-14, pP1-570, and pHP-3a) used to generate
the HCo7 transgene is outlined below.

5   pH3V4D.

Germline configuration heavy chain variable gene
segments were isolated from phage 1 genomic DNA libraries using
synthetic oligonucleotide probes for VH1 and VH3 classes. The
VH1 class probe was o-49:

10   5'- gtt aaa gag gat ttt att cac ccc tgt gtc ctc tcc aca ggt gtc
     -3'

The VH3 class probe was o-184:
     5'- gtt tgc agg tgt cca gtg t(c,g)a ggt gca gct g(g,t)t gga gtc
15   (t,c)(g,c)g -3'

Positively hybridizing clones were isolated,
partially restriction mapped, subcloned and partially
sequenced. From the nucleotide sequence it was determined that
20   one of the VH1 clones isolated with the o-49 probe encoded a VH
gene segment, 49.8, comprising an amino acid sequence identical
to that contained in the published sequence of the hv1263 gene
(Chen et al. (1989) Arthritis Rheum. 32: 72). Three of the VH3
genes, 184.3, 184.14, and 184.17, that were isolated with the
25   o-184 probe contained sequences encoding identical amino acid
sequences to those contained in the published for the VH genes
DP-50, DP-54, and DP-45 (Tomlinson et al. (1992) J. Mol. Biol.
227: 776). These four VH genes were used to build the pH3V4D
plasmid.

30   The 184.3 gene was found to be contained within a 3
kb BamHI fragment. This fragment was subcloned into the
plasmid vector pGP1f such that the XhoI site of the polylinker

is 5' of the gene, and the SalI site is 3'. The resulting

plasmid is called p184.3.36f. The 184.14 gene was found to be

contained within a 4.8 kb HindIII fragment. This fragment was

subcloned into the plasmid vector pUC19 in an orientation such

5   that the gene could be further isolated as a 3.5 kb fragment by

XhoI/SalI digestion at a genomic XhoI site 0.7 kb upstream of

the gene and a polylinker derived SalI site 3' of the gene.

The resulting plasmid is called p184.14.1. The 184.17 gene was

found to be contained within a 5.7 kb HindIII fragment. This

10   fragment was subcloned into the plasmid vector pSP72 (Promega,

Madison, WI) in an orientation such that the polylinker derived

XhoI and SalI sites are, respectively, 5' and 3' of the gene.

The insert of this plasmid includes an XhoI site at the 3' end

of the gene which was eliminated by partial digestion with

15   XhoI, Klenow fragment filling-in, and religation. The

resulting plasmid is called p184.17SK. The 49.8 gene was found

to be contained within 6.3 kb XbaI fragment. This fragment was

subcloned into the plasmid vector pNN03, such that the

polylinker derived XhoI and ClaI sites are, respectively, 5'

20   and 3' of the gene, to create the plasmid pVH49.8 (Taylor et

al. (1994) *International Immunol.* 6: 579). The XhoI/ClaI

insert of pVH49.8 was then subcloned into pGP1f to create the

plasmid p49.8f, which includes unique XhoI and SalI sites

respectively at the 5' and 3' end of the 49.8 gene.

25   The 3.5 kb XhoI/SalI fragment of p184.14.1 was cloned

into the XhoI site of p184.3.36f to generate the plasmid

pRMVH1, which includes both the 184.14 and the 184.3 genes in

the same orientation. This plasmid was digested with XhoI and

the 5.7 kb XhoI/SalI fragment of p184.17SK was inserted to

30   create the plasmid pRMVH2, which contains, from 5' to 3', the

three VH genes 184.17, 184.14, and 184.3, all in the same

orientation. The plasmid pRMVH2 was then cut with XhoI, and

the 6.3 kb XhoI/SalI insert of p49.8f inserted to create the plasmid pH3VH4, which contains, from 5' to 3', the four VH genes 49.8, 184.17, 184.14, and 184.3, all in the same orientation.

5   The 10.6 kb XhoI/EcoRV insert of the human D region clone pDH1 (described supra; e.g., in Example 12) was cloned into XhoI/EcoRV digested pGPe plasmid vector to create the new plasmid pDH1e. This plasmid was then digested with EcoRV and ligated with a synthetic linker fragment containing a SalI site

10 (5'- ccg gtc gac ccg -3'). The resulting plasmid, pDH1es, includes most of the human D1 cluster within an insert that can be excised with XhoI and SalI, such that the XhoI site is on the 5' end, and the SalI site is on the 3' end. This insert was isolated and cloned into the SalI site of pH3VH4 to create

15 the plasmid pH3VH4D, which includes four germline configuration human VH gene segments and 8 germline configuration human D segments, all in the same 5' to 3' orientation. The insert of this clone can be isolated, substantially free of vector sequences, by digestion with NotI.

20

pCOR1xa

          The plasmid pCOR1 (described supra) which contains a 32 kb XhoI insert that includes 9 human D segments, 6 human J segments, the $J\mu$ intronic heavy chain enhancer, the μ switch

25 region, and the $C\mu$ coding exons--was partially digested with XhoI, Klenow treated, and a synthetic SalI linker ligated in to produce the new plasmid pCOR1xa, which has a unique XhoI site at the 5' end and a unique SalI site at the 3' end. Both pCOR1 and pCOR1xa contain a 0.6 kb rat heavy chain 3' enhancer

30 fragment at the 3' end, which is included in the insert if the plasmid is digested with NotI instead of XhoI or XhoI/SalI.

pP1-570

A phage P1 library (Genome Systems Inc., St. Louis, Missouri) was screened by PCR using the oligonucleotide primer pair:

5   5'- tca caa gcc cag caa cac caa g -3'

5'- aaa agc cag aag acc ctc tcc ctg -3'

This primer pair was designed to generate a 216 bp PCR product with a human γ gene template. One of the P1 clones identified

10 was found to contain both the human γ3 and γ1 genes within an 80 kb insert. The insert of this clone, which is depicted in Fig. 80, can be isolated, substantially free of vector sequences, by digestion with NotI and SalI.

15 p11-14

Restriction mapping of the human γ3/γ1 clone P1-570 revealed a 14 kb BamHI fragment near the 5' end of the insert. This 14 kb fragment was subcloned into the plasmid vector pGP1f such that the polylinker derived SalI site is adjacent to the

20 5' end of the insert. The resulting plasmid is called pB14. Separately, an 11 kb NdeI/SpeI genomic DNA fragment covering the 3' end of the human μ gene and the 5' end of the human δ gene, derived from the plasmid clone pJ1NA (Choi et al. (1993) Nature Genetics 4: 117), was subcloned into the SalI site of

25 pBluescript (Stratagene, LaJolla, CA) using synthetic oligonucleotide adapters. The resulting SalI insert was then isolated and cloned into the SalI site of pB14 such that the relative 5' to 3' orientation of the μ fragment from pJ1NA is the same as that of the γ fragment from P1-570. The resulting

30 clone is called p11-14. The insert of this clone can be isolated, substantially free of vector sequences, by digestion with NotI.

pHP-3a

The mouse heavy chain 3' enhancer (Dariavach et al.
(1991) Eur. J. Immunol. 21: 1499; Lieberson et al. (1991)
Nucleic Acids Res. 19: 933) was cloned from a balb/c mouse
5   genomic DNA phage λ library. To obtain a probe, total balb/c
mouse thymus DNA was used as a template for PCR amplification
using the following two oligonucleotides:

cck76:   5'- caa tag ggg tca tgg acc c -3'
10   cck77:   5'- tca ttc tgt gca gag ttg gc -3'

The resulting 220 bp amplification product was cloned
using the TA Cloning™ Kit (Invitrogen, San Diego, CA) and the
insert used to screen the mouse phage library. A positively
15   hybridizing 5.8 kb HindIII fragment from one of the resultant
phage clones was subcloned into pGP1f. The orientation of the
insert of this subclone, pHC3'ENfa, is such that the
polylinker XhoI site is adjacent to the 5' end of the insert
and the SalI site adjacent to the 3' end. Nucleotide sequence
20   analysis of a portion of this HindII fragment confirmed that it
contained the 3' heavy chain enhancer. The insert of pHC3'ENfa
includes an XhoI site approximately 1.9 kb upstream of the
EcoR1 site at the core of the enhancer sequence. This XhoI
site was eliminated by partial digestion, Klenow fill-in, and
25   religation, to create the clone pH3'Efx, which includes unique
XhoI and SalI sites, respectively, at the 5' and 3' ends of the
insert.

The 3' end of the human γ3/γ1 clone P1-570 was
subcloned as follows: P1-570 DNA was digested with NotI,
30   klenow treated, then digested with XhoI; and the 13 kb end
fragment isolated and ligated to plasmid vector pGP2b which had
been digested with BamHI, klenow treated, and then digested

with XhoI. The resulting plasmid, pPX-3, has lost the polylinker NotI site adjacent to the polylinker XhoI site at the 5' end of the insert; however, the XhoI site remains intact, and the insert can be isolated by digestion with NotI

5   and XhoI, or SalI and XhoI. The 3' enhancer containing XhoI/SalI insert of pH3'Efx was isolated and ligated into the 3' SalI site of pPX-3 to create the plasmid pHP-3a. The enhancer containing fragment within the pHP-3a insert is ligated in the opposite orientation as the 3' end of the P1-570

10 clone. Therefore, pHP-3a contains an internal SalI site, and the insert is isolated by digestion with XhoI and NotI. Because this is an enhancer element, 5' to 3' orientation is generally not critical for function.

15 HCo7.

       To prepare the HCo7 DNA mixture for pronuclear microinjection, DNA from each of the five plasmids described above was digested with restriction enzymes and separated on an agarose gel. Clone pH3V4D was cut with NotI; pCOR1xa was cut

20 with NotI; p11-14 was cut with NotI; pP1-570 was cut with NotI and SalI; and pHP-3a was cut with NotI and XhoI. The DNA inserts were electroeluted and further purified on an equilibrium CsCl gradient without EtBr. The inserts were dialyzed into injection buffer and mixed as follows: 50

25 microliters of pH3V4D insert @ 20.4 ng/microliter; 50 microliters of pCOR1xa insert @ 20.8 ng/microliter; 50 microliters of p11-14 insert @ 15.6 ng/microliter; 300 microliters of pP1-570 insert @ 8.8 ng/microliter; 60 microliters of pHP-3a insert @ 10.8 ng/microliter; and 1.49 ml

30 injection buffer.

HCo7 transgenic animals

The HCo7 DNA mixture was microinjected into the
pronuclei of one-half day old embryos, and the embryos
transferred into the oviducts of pseudopregnant females, as
described by Hogan et al. (Manipulating the mouse embryo, Cold
5   Spring Harbor laboratories, Cold Spring Harbor NY).

Tail tip DNA was isolated from 202 animals that
developed from microinjected embryos. Southern blot analysis
of this DNA, using a probe comprising human μ and DH sequences,
revealed 22 founder animals that had incorporated at least a
10   portion of the HCo7 transgene. Fig. 81 shows an analysis of
the expression of human μ and human $\gamma 1$ in the serum of 6 G0
animals that developed from embryos microinjected with HCo7
DNA. Serum levels of human immunoglobulin proteins were
measured by ELISA as described in Lonberg et al. (1994) Nature
15   368: 856. Four of these six mice showed evidence of
incorporation of the transgene by Southern blot analysis, and
three of these mice expressed both human μ and human $\gamma 1$
proteins in their serum. The single transgenic mouse that did
not express human immunoglobulin proteins was determined by
20   Southern blot analysis to contain only a low number of copies
of the transgene, and it is possible that the entire transgene
was not incorporated, or that this mouse was a genetic mosaic.
Two of the founder HCo7 mice, #11952 and #11959, were bred with
human κ minilocus (KCo4 line 4436) transgenic mice that were
25   also homozygous for disruptions of the endogenous heavy, and κ
light chain loci (Lonberg et al. op.cit), to generate mice that
were homozygous for the two endogenous locus disruptions and
hemizygous for the two introduced human miniloci, KCo4 and
HCo7. Five of these so-called
30   double-transgenic/double-deletion mice were analyzed for
expression of human IgM, human IgG1, and human IgG3. As a
control, three HC2/KCo4 double-transgenic/double-deletion mice

were included in the analysis. This experiment is presented in
Fig. 82. The ELISA data in this figure was collected as in
Lonberg et al. (*op.cit*), except that for detection of human
IgG3, the coating antibody was a specific mAb directed against

5   human IgG3 (cat. # 08041, Pharmingen, La Jolla, CA); the other
details of the IgG3 assay were identical to those published for
IgG1. While the HC2/KCo4 mice express only human IgM and human
IgG1, the HCo7/KCo4 mice also express human IgG3 in addition to
these two isotypes. Expression of human γ3 and γ1 in the HCo7

10   mice has also been detected by PCR amplification of cDNA
synthesized from RNA isolated from the spleen of a transgenic
mouse. Fig. 83 depicts PCR amplification products synthesized
using spleen cDNA from three different lines of transgenic
mice: line 2550 is an HC2 transgenic line, while lines 11959

15   and 11952 are HCo7 transgenic lines. Single stranded cDNA was
synthesized from spleen RNA as described by Taylor et al.
(1992) Nucleic Acid Res. 20: 6287. The cDNA was then PCR
amplified using the following two oligonucleotides:

20   o-382:   5'- gtc cag aat tcg gt(c,g,t) cag ctg gtg (c,g)ag tct
gg -3'

     o-383:   5'- ggt ttc tcg agg aag agg aag act gac ggt cc -3'

25   This primer pair directs the synthesis of PCR products that
spans the hinge region of human γ transcripts. Because of
differences in the structures of the human γ1 and γ3 hinge
regions, PCR amplification distinguishes between these two
transcripts. A human γ1 template will direct the synthesis of

30   a 752 bp PCR product, while human γ3 directs the synthesis of a
893 bp product. While only human γ1 template is detectable in
the HC2 line 2550 and HCo7 line 11959 spleens, both γ1 and γ3

transcripts are detectable in the HCo7 line 11952 spleen.
Because of the non-quantitative nature of this assay, and
because of differences in $\gamma 3$ expression between individual
animals (shown by ELISA in Fig. 82), the inability to observe

5    $\gamma 3$ in the HCo7 line 11959 spleen in Fig. 83 does not indicate
that $\gamma 3$ is not expressed in this line. Isolated spleen cells
from the HCo7/KCo4 mice can also be induced to express both
IgG1 and IgG3 _in vitro_ by stimulation with LPS and IL4. This
experiment is shown in Fig. 84. Spleen cells from a 7 week old

10   male HCo7/KCo4 double-transgenic/double-deletion mouse (#12496;
line 11959/4436) tested for immunoglobulin secretion in
response to the thymus-independent B cell mitogen, LPS, alone
and in conjunction with various cytokines. Splenocytes were
enriched for B cells by cytotoxic elimination of T cells.

15   B-enriched cells were plated in 24 well plates at 2×10^6 cells
per well in 2 ml of 10% FCS in RPMI-1640. LPS was added to all
wells at 10 micrograms/ml. IL-2 was added at 50 units/ml, IL-4
was added at 15 ng/ml, IL-6 was added at 15 ng/ml, γIFN was
added at 100 units/ml. Cultures were incubated at 37°C, 5% CO_2

20   for 10 days, then supernatants were analyzed for human IgG1 and
IgG3 by ELISA. All reagents for ELISA were polyclonal
anti-serum from Jackson Immunologicals (West Grove, PA), except
the capture anti-human IgM, which was a monoclonal antibody
from The Binding Site (Birmingham, UK).

25

EXAMPLE 38

This example demonstrates the successful introduction
into the mouse genome of functional human light chain V
segments by co-injection of a human κ light chain minilocus and

30   a YAC clone comprising multiple human V_κ segments. The example
shows that the V_κ segment genes contained on the YAC contribute
to the expressed repertoire of human κ chains in the resultant

mouse. The example demonstrates a method for repertoire
expansion of transgene-encoded human immunoglobulin proteins,
and specifically shows how a human κ chain variable region
repertoire can be expanded by co-introduction of unlinked
5   polynucelotides comprising human immunoglobulin variable region
segments.

Introduction of functional human light chain V segments by
co-injection of Vk containing yeast artificial chromosome clone
10   DNA and k light chain minilocus clone DNA

          I. Analysis of a yeast strain containing cloned human Vk
gene segments.

          Total genomic DNA was isolated from a yeast strain
containing a 450 kb yeast artificial chromosome (YAC)
15   comprising a portion of the human V_κ locus (ICRF YAC library
designation 4x17E1). To determine the identity of some of the
V_κ gene segments included in this YAC clone, the genomic DNA was
used as a substrate for a series of V_κ family specific PCR
amplification reactions. Four different 5' primers were each
20   paired with a single consensus 3' primer in four sets of
amplifications. The 5' primers were: o-270 (5'-gac atc cag ctg
acc cag tct cc-3'), o-271 (5'-gat att cag ctg act cag tct
cc-3'), o-272 (5'-gaa att cag ctg acg cag tct cc-3'), and o-273
(5'-gaa acg cag ctg acg cag tct cc-3'). These primers are used
25   by Marks et al. (Eur. J. Immunol. 1991. 21, 985) as V_κ family
specific primers. The 3' primer, o-274 (5'-gca agc ttc tgt ccc
aga ccc act gcc act gaa cc-3'), is based on a consensus
sequence for FR3. Each of the four sets of primers directed
the amplification of the expected 0.2 kb fragment from yeast
30   genomic DNA containing the YAC clone 4x17E1. The 4 different
sets of amplification products were then gel purified and
cloned into the PvuII/HindIII site of the plasmid vector pSP72

(Promega). Nucleotide sequence analysis of 11 resulting clones
identified seven distinct V genes. These results are presented
below in Table 14.

5   Table 14.   Identification of human V$_\kappa$ segments on the YAC
     4x17E1.

PCR primers	clone #	identified gene	Vκ family
o-270/o-274	1	L22*	I
- " -	4	L22*	I
- " -	7	O2* or O12	I
o-271/o-274	11	A10*	VI
- . -	15	A10*	VI
o-272/o-274	20	A4* or A20	I
- . -	21	A11*	III
- . -	22	A11*	III
- . -	23	A11*	III
- . -	25	O4* or O14	I
o-273/o-274	36	L16* or L2	III

* Gene segments mapped within the distal V$_\kappa$ cluster (Cox et al.
Eur. J. Immunol. 1994. 24, 827; Pargent et al. Eur. J. Immunol.
1991. 21, 1829; Schable and Zachau Biol. Chem. Hoppe-Seyler
1993. 374, 1001)

        All of the sequences amplified from the YAC clone are
either unambiguously assigned to V$_\kappa$ genes that are mapped to the
distal cluster, or they are compatible with distal gene
sequences. As none of the sequences could be unambiguously
assigned to proximal V genes, it appears that the YAC 4x17E1
includes sequences from the distal Vκ region. Furthermore, one
of the identified sequences, clone #7 (VkO2), maps near the J
proximal end of the distal cluster, while another sequence,

clones # 1 and 4 (VkL22), maps over 300 kb upstream, near the J

distal end of the distal cluster. Thus, if the 450 kb YAC

clone 4x17E1 represents a non-deleted copy of the corresponding

human genome fragment, it comprises at least 32 different V_κ

5   segments. However, some of these are non-functional

pseudogenes.

         2.   Generation of transgenic mice containing YAC

derived V_κ gene segments.

10   To obtain purified YAC DNA for microinjection into

embryo pronuclei, total genomic DNA was size fractionated on

agarose gels. The yeast cells containing YAC 4x17E1 were

imbedded in agarose prior to lysis, and YAC DNA was separated

from yeast chromosomal DNA by standard pulse field gel

15   electrophoresis (per manufacturers specifications: CHEF DR-II

electrophoresis cell, BIO-RAD Laboratories, Richmond CA). Six

individual pulse field gels were stained with ethidium bromide

and the YAC clone containing gel material was cut away from the

rest of the gel. The YAC containing gel slices were then

20   imbedded in a new (low melting temperature) agarose gel cast in

a triangular gel tray. The resulting triangular gel was

extended at the apex with a narrow gel containing two

moles/liter sodium acetate in addition to the standard gel

buffer (Fig. 85).

25   The gel was then placed in an electrophoresis chamber

immersed in standard gel buffer. The "Y"-shaped gel former

rises above the surface of the buffer so that current can only

flow to the narrow high salt gel slice. A Plexiglas block was

placed over the high salt gel slice to prevent diffusion of the

30   NaOAc into the gel buffer. The YAC DNA was then

electrophoresed out of the original gel slices and into the

narrow high salt block. At the point of transition from the

low salt gel to the high salt gel, there is a resistance drop
that effectively halts the migration of the YAC DNA through the
gel. This leads to a concentration of the YAC DNA at the apex
of the triangular gel. Following electrophoresis and staining,
5 the concentrated YAC DNA was cut away from the rest of the DNA
and the agarose digested with GELase (EPICENTRE Technologies).
Cesium chloride was then added to the YAC DNA containing liquid
to obtain a density of 1.68 g/ml. This solution was
centrifuged at 37,000 rpm for 36 hrs to separate the DNA from
10 contaminating material. 0.5 ml fractions of the resulting
density gradient were isolated and the peak DNA containing
fraction dialyzed against 5 mM tris (pH 7.4)/5 mM NaCl/0.1 M
EDTA. Following dialysis, the concentration of the resulting
0.65 ml solution of YAC DNA was found to be 2 micrograms/ml.
15 This DNA was mixed with purified DNA insert from plasmids pKC1B
and pKV4 (Lonberg et al. 1994. Nature 368, 856) at a ratio of
20: 1: 1 (micrograms YAC4x17E1: KC1B: KV4). The resulting 2
microgram/ml solution was injected into the pronuclei of
half-day mouse embryos, and 95 surviving microinjected embryos
20 transferred into the oviducts of pseudo-pregnant females.
Thirty nine mice were born that developed from the
microinjected embryos. Two of these mice, #9269 and #9272,
were used to establish transgenic lines. The lines are
designated KCo5-9269 and KCo5-9272.
25   A Southern blot analysis of genomic DNA from mice of
lines KCo5-9269 and KCo5-9272 was carried out to determine if
YAC 4x17E1 derived V_κ segments had been incorporated in their
genomes. A V_κ gene segment, VkA10 (accession #: x12683;
Straubinger et al. 1988. Biol. Chem. Hoppe-Seyler 369,
30 601-607), from the middle of the distal V_κ cluster was chosen as
a probe for the Southern blot analysis. To obtain the cloned
probe, the VkA10 gene was first amplified by PCR. The two

oligo nucleotides, o-337 (5'- cgg tta aca tag ccc tgg gac gag
ac -3') and o-338 (5'- ggg tta act cat tgc ctc caa agc ac -3'),
were used as primers to amplify a 1 kb fragment from YAC
4x17E1.   The amplification product was gel purified, digested

5   with HincII, and cloned into pUC18 to obtain the plasmid
p17E1A10. The insert of this plasmid was then used to probe a
southern blot of KCo5-9269 and KCo5-9272 DNA. The blot showed
hybridization of the probe to the expected restriction
fragments in the KCo5-9272 mouse DNA only. This indicates that

10   the VkA10 gene is incorporated into the genome of KCo5-9272
mice and not KCo5-9269 mice. Line KCo5-9272 mice were then
bred with HC2-2550/JHD/JKD mice to obtain mice homozygous for
disruptions of the endogenous heavy and κ light chain loci, and
hemi- or homozygous for the HC2 and KCo5 transgenes. Animals

15   that are homozygous for disruptions of the endogenous heavy and
k light chain loci, and hemi- or homozygous for human heavy and
k light chain transgenes are designated double
transgenic/double deletion mice.

A cDNA cloning experiment was carried out to

20   determine if any of the YAC-derived V_x genes are expressed in
line KCo5-9272 mice. The double transgenic/double deletion
mouse #12648 (HC2-2550/KCo5-9272/JHD/JKD) was sacrificed and
total RNA isolated from the spleen. Single stranded cDNA was
synthesized from the RNA and used as a template in four

25   separate PCR reactions using oligonucleotides o-270, o-271,
o-272, and o-273 as 5' primers, and the Ck specific
oligonucleotide, o-186 (5'- tag aag gaa ttc agc agg cac aca aca
gag gca gtt cca -3'), as a 3' primer. The amplification
products were cloned into the pCRII TA cloning vector

30   (Invitrogen). The nucleotide sequence of 19 inserts was
determined. The results of the sequence analysis are
summarized in Table 15 below.

Table 15.   Identification of human Vk genes expressed in mouse line KCo5-9272.

	PCR primers	clone #	identified gene	Vk family
5	o-270/o-186	1	L15*	I
	- .-	3	L18**	I
	- .-	7	L24**	I
	- .-	9	L15*	I
	- .-	10	L15*	I.
10	o-271/o-186	15	A10**	VI
	- .-	17	A10**	VI
	- .-	18	A10**	VI
	- .-	19	A10**	VI
	- .-	21	A10**	VI
15	o-272/o-186	101	A27*	III
	- .-	102	L15*	I
	- .-	103	A27*	III
	- .-	104	A27*	III
	o-273/o-186	35	A27*	III
20	- .-	38	A27*	III
	- .-	44	A27*	III
	- .-	45	A27*	III
	- .-	48	A27*	III

25   * Vk genes encoded by transgene plasmid sequences.

** Vk genes encoded uniquely by YAC derived transgene sequences.

These results show that at least 3 of the YAC derived V_x gene segments, A10, L18, and L24, contribute to 30 the expressed human repertoire of the line KCo5-9272 mice.

To determine the effect of this increased repertoire on the size of the various B220⁺ cell populations in the bone marrow and spleen, a flow 35 cytometric analysis was carried out on line KCo5-9272 mice. Part of this analysis is shown in Figs. 86 and 87.

Two double transgenic/double deletion mice, one
containing the KCo5 transgene, and one containing the
KCo4 transgene, are compared in this experiment. These
two transgenes share the same joining and constant region
5   sequences, as well as the same intronic and 3' enhancer
sequences. They also share four different cloned V gene
segments; however, the KCo5 transgene includes the
additional V segments derived from YAC 4x17E1 that are
not included in the KCo4 transgene. Cells were isolated
10   from mouse #13534 (HC2-2550/KCo5-9272/JHD/JKD) and mouse
#13449 (HC2-2550/KCo4-4436/JHD/JKD). Bone marrow cells
were stained with anti-mouse B220 (Caltag, South San
Francisco, CA), anti-mouse CD43 (Pharmingen, La Jolla,
CA), and anti-human IgM (Jackson Immunologic, West Grove,
15   PA). Spleen cells were stained with anti-mouse B220 and
anti-human IgM.

Fig. 86 shows a comparison of the B cell, and B
cell progenitor populations in the bone marrow of KCo5
and KCo4 mice. The fraction of B cells in the bone
20   marrow (B220+, IgM+) is approximately three times higher
in the KCo5 mice (6%) than it is in the KCo4 mice (2%).
The pre-B cell population (B220+, CD43-, IgM-) is also
higher in the KCo5 mice (9%, compared to 5% for KCo4).
Furthermore, the pro-B compartment (B220+, CD43+) is
25   elevated in these mice (11% for KCo5 and 5% for KCo4).
Although each of these three compartments is larger in
the KCo5 mice than it is in the KCo4 mice, the levels are
still approximately half that found in wild type mice.
The increase in the number of bone marrow B cells is
30   presumably a direct consequence of the increased
repertoire size. The larger primary repertoire of these
mice may provide for membrane Ig with some minimal

threshold affinity for endogenous antigens. Receptor
ligation could then allow for proliferation of those B
cells expressing the reactive Ig. However, because the
pre-B and pro-B cells do not express light chain genes,

5   the explanation for the increased sizes of these two
compartments in the KCo5 mice is not immediately
apparent. The B cell progenitor compartments may be
larger in KCo5 mice because the increased number of B
cells creates a bone marrow environment that is more

10   conducive to the expansion of these populations. This
effect could be mediated directly by secreted factors or
by cell-cell contact between B cells and progenitor
cells, or it could be mediated indirectly, by titration
of factors or cells that would otherwise inhibit the

15   survival or proliferation of the progenitor cells.

Fig. 87 shows a comparison of the splenic B
cell (B220$^+$, IgM$^+$) populations in KCo5 and KCo4 mice. The
major difference between these two mice is the relative
sizes of B220^{dull} B cell populations (6% in the KCo5 mice

20   and 13% in the KCo4 mice). The B220^{dull} cells are larger
than the B220^{bright} B cells, and a higher fraction of them
express the l light chain. These are characteristics of
the so-called B1 population that normally dominates the
peritoneal B cell population in wild type mice. The

25   spleens of the KCo4 mice comprise an anomolously high
fraction of B220^{dull} cells, while the KCo5 mice have a more
normal distribution these cells. However, both strains
contain approximately one-half to one-third the normal
number of B cells in the spleen.

30

EXAMPLE 39

This example demonstrates the successful use of KCo5 transgenic mice of Example 38 to isolate hybridoma clones that secrete high affinity, antigen specific, human IgG monoclonal antibodies.

Immunization. A double deletion/double transgenic mouse (KCo5-9272/HC2-2550/JHD/JKD, #12657) was immunized intraperitoneally every other week for eight weeks with 4 to 10 x 10^6 irradiated T4D3 cells, a murine T cell line expressing human CD4 (Dr. Jane Parnes, Stanford University) followed by one injection intraperitoneally two weeks later of 20 mg soluble recombinant human CD4 (sCD4; Intracell) in incomplete Fruend's adjuvant (Sigma). The mouse was boosted once 3 days prior to fusion with 20 mg sCD4 intravenously.

Hybridoma fusion. Single cell suspensions of splenic lymphocytes from the immunized mouse were fused to one-sixth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (ATCC CRL 1580) with 50% PEG (Sigma). Cells were plated at approximately 2 X 10^5 in flat bottom microtiter plates, followed by a two week incubation in selective medium containing 20% Fetal Clone Serum (HyClone), 18% "653" conditioned medium, 5% Origen (IGEN), 4 mM L-glutamine, 1 mM sodium pyruvate, 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml mM penicillin, 50 mg/ml streptomycin, 50 mg/ml mM gentamycin and 1X HAT (Sigma; the HAT was added 24 hrs after the fusion). After two weeks, cells were cultured in medium in which the HAT was replaced with HT. Wells were screened by ELISA and flow cytometry once extensive hybridoma growth or spent medium was observed.

Hybridoma screening by ELISA. To detect anti-CD4 mAbs, microtiter plates (Falcon) were coated overnight at 4°C with 50 ml of 2.5 mg/ml of sCD4 in PBS, blocked at RT for 1 hr with 100 ml of 5% chicken serum in PBS, and then sequentially incubated at RT for 1 hr each with 1:4 dilutions of supernatant from hybridomas, 1:1000 dilution of F(ab')$_2$ fragments of horseradish peroxidase (HRPO)-conjugated goat anti-human IgG (Jackson) or 1:250 dilution of HRPO-conjugated goat anti-human Igk antibodies (Sigma) plus 1% normal mouse serum, and finally with 0.22 mg/ml ABTS in 0.1 M citrate phosphate buffer, pH 4 with 0.0024% H_2O_2. Plates were washed 3-6 times with wash buffer (0.5% Tween-20 in PBS) between all incubations, except the first. Diluent (wash buffer with 5% chicken serum) was used to dilute the supernatants and the HRPO conjugates. Absorbance was measured using dual wavelengths (OD at the reference wavelength of 490 nm was subtracted from the OD at 415 nm).

To detect mouse λ-containing mAbs, the above ELISA protocol was used, with the following exceptions. Wells of microtiter plates were coated with 100 ml of 1) 1.25 mg/ml goat anti-mouse λ (Pierce), 2) 1.25 mg/ml goat anti-human Fcγ (Jackson), or 3) 2.5 mg/ml sCD4 (ABT). For the detection step, 100 ml of 1:5000 goat anti-mouse l (SBA) conjugated to biotin was used followed by 100 ml of 1:1000 streptavidin conjugated to HRPO (Jackson). Murine and human mAb standards were used at the indicated concentrations. To look for cross-reactivity to unrelated antigens, wells were coated with CEA (Crystal Chem), KLH (CalBiochem), HSA (Sigma), BSA (Sigma) or OVA (Sigma; all at 2 mg/ml, except CEA which was at 2.5). Appropriate antibodies were titered and used as positive

controls (human IgM anti-CEA (GenPharm), rabbit anti-KLH
(Sigma), sheep anti-HSA (The Binding Site), sheep
anti-BSA (The Binding Site), and sheep anti-OVA (The
Binding Site)). Any bound antibody was detected with

5   HRPO conjugates of goat anti-human IgM, donkey
anti-rabbit IgG or donkey anti-sheep IgG (all diluted
1:1000 and obtained from Jackson). Otherwise, the
standard ELISA protocol was followed.

10   Hybridoma screening by flow cytometric assay. To further
screen for mAbs reactive with native cell-surface CD4, 5
x 10^5 SupT1 cells (ATCC CRL 1942) were incubated on ice
with a 1:2 dilution of spent supernatant from the fusion
plates for 30 min, washed twice with cold stain buffer

15   (0.1% BSA, 0.02% NaN$_3$ in PBS), incubated with 1.5 mg/ml of
an F(ab')$_2$ fragment of FITC-conjugated goat anti-human Fcg
(FITC-GaHuIgG; Jackson) for 15 min, washed once and
analyzed immediately on a FACScan (Becton-Dickinson).

20   CD4 reactive hybridomas. Using the ELISA and flow
cytometric techniques described above, 12 hybridoma
clones were identified that secreted human IgG
specifically reactive with native human CD4. Ten of
these twelve clones were further subcloned. Eight of

25   these subclones were identified as human IgG1κ secreting
hybridomas. The other two expressed a mouse λ light
chain. The parent wells for the 8 fully human clones
were: 1E11, 2E4, 4D1, 6C1, 6G5, 7G2, 10C5, and 1G1. Flow
cytometric assays of the binding of 3 of the fully human

30   IgGk subclones (4D1.4, 6G5.1, and 10C5.6) are shown in
Fig. 88.

Fig. 88 shows binding of IgGκ anti-nCD4
monoclonal antibodies to CD4+ SupT1 cells. Cells from
log phase growth cultures were washed and stained with no
monoclonal antibody, 4E4.2 (as a negative control),
chimeric Leu3a (as a positive control), or with one of
the 10 human IgG anti-nCD4 monoclonal antibodies. Any
bound monoclonal antibody was detected with
FITC-conjugated goat anti-human Fcγ. All ten monoclonal
antibodies bound to SupT1 cells, although data is shown
here for only three of them.

Analysis of human antibody secretion by cloned
hybridomas. To compare the growth and secretion levels
of mAbs, the subclones were put into replicate cultures
in HT medium in 24 well plates at an initial density of 2
x 10^5 cells/ml. Each day for 7 days, one of the replicate
cultures for each subclone was harvested and cell
numbers, cell viability (by Trypan blue exclusion) and
the amount of mAb in the supernatant (by a quantitative
ELISA for total human γ) were determined. Table 16 shows
data for antibody secretion by 7 of the hybridoma
subclones.

Table 16. Secretion Levels For Human IgGk Anti-nCD4
Monoclonal Antibodies

Subclone	pg/cell	pg/cell/d
1E11.15	3.9	0.56
1G1.9	11	1.5
4D1.4	1.4	0.91
6C1.10	3.3	0.48
6G5.1	7.8	1.1
7G2.2	4.4	0.63
10C5.6	8.0	1.1

* pg/cell = (maximum amount of mAb)/(maximum number of viable cells)   pg/cell/d = (pg/cell)/7 days

Purification of human mAbs. The individual hybridoma clones were grown in medium without HT and Origen and the FCS was gradually decreased to approximately 2-3% in the final 1 l cultures. Supernatants were harvested once the viability of the hybridomas fell below approximately 30%. To purify the IgGk mAbs, the spent supernatants were centrifuged to remove cells, concentrated via ultrafiltration to approximately 50 to 100 mls, diluted 1:5 with PBS, pH 7.4 and loaded onto a 5 ml Protein A (Pharmacia) column. After washing with 3-5 column volumes of PBS, the human IgGk mAbs were eluted with 0.1 HCl, 150 mM NaCl, pH 2.8 and immediately neutralized with 1M Tris base. Column fractions containing material with an OD_{280} > 0.2 were pooled and dialyzed into PBS. The OD_{280} was then determined and an absorbtivity coefficient of 1.4 was used to calculate the protein concentration of the human IgG. No mAb was detected in the flow through and the % recoveries ranged from 93 to 100%. Three to six mgs of each purified mAb were obtained, with >90% purity.

Analysis of monoclonal antibodies from cloned hybridomas. To investigate the specificity of binding of mAbs, human PBMC were isolated over Ficoll and stained as follows. Human PBMC (10^6) in stain buffer were incubated for 30 min on ice, in separate reactions, with equal volumes of supernatant from each of three of the subcloned hybridomas (4D1.4, 6G5.1, and 10C5.6), or with an isotype

matched negative control mAb, washed twice, and incubated

20 min on ice with 1 mg/ml of FITC-GaHuIgG along with

either 10 ml of mouse anti-human CD4 mAb (Leu3a;

Becton-Dickinson) conjugated to phycoerythrin (PE), 10 ml

5   of mouse anti-human CD8 mAb (Leu2a; Becton-Dickinson)

conjugated to PE, or 5 ml of mouse anti-human CD19 mAb

(SJ25-C1; Caltag) conjugated to PE. Gated lymphocytes

were then analyzed on a FACScan flow cytometer (Becton

Dickinson, San Jose, CA). All three of the antibodies

10   were found to bind specifically to the CD4 fraction of

the human PBMC.

To approximate the location of the epitope

recognized by these three mAbs, 5 x 10^5 SupT1 cells were

pre-incubated for 20 min on ice with buffer, 2.5 mg/ml

15   RPA-T4, or 2.5 mg/ml Leu3a in stain buffer, then for 30

min with one of the 10 human IgG mAbs (in supernatant

diluted 1:2) and finally with 0.5 mg/ml FITC-conjugated

goat anti-human Fcγ to detect any bound human IgG. Cells

were washed twice with stain buffer prior to and once

20   after the last step. The results of this blocking assay

are shown in Fig. 89. None of the three antibodies share

an epitope with RPA-T4, while 6G5.1 and 10C5.6 appear to

recognize the same (or an adjacent) epitope as that

recognized by Leu3a.

25

Rate and equilibrium constant determinations.

Human sCD4 (2500 to 4200 RU) was immobilized by

covalent coupling through amine groups to the sensor chip

surface according to manufacturer's instructions.

30   Antibody dilutions were flowed over the antigen-coupled

sensor chips until equilibrium was reached, and then

buffer only was allowed to flow. For each phase of the

reaction, binding and dissociation, the fraction of bound
antibody was plotted over time. The derivative of the
binding curve (dR/dt) was calculated and plotted against
the response for each concentration. To calculate the

5   association rate constant (k_{assoc}), the slopes of those
resulting lines were then plotted against the
concentration of the monoclonal antibody. The slope of
the line from this graph corresponded to the k_{assoc}. The
dissociation rate constant (k_{dissoc}) was calculated from the

10   log of the drop in response (during the buffer flow
phase) against the time interval. The Ka was derived by
dividing the k_{assoc} by the k_{dissoc}. The measured rate and
affinity constant data for 5 different purified
monoclonal antibodies derived from the KCo5/HC2 double

15   transgenic/double deletion mice, and one purified
antibody obtained from a commercial source (Becton
Dickinson, San Jose, CA), is presented in Table 17.

Table 17. Rate and affinity constants for monoclonal
20   antibodies that bind to human CD4.

Hybridoma	Antibody	Source	k_{ass} $(M^{-1}s^{-1})$	k_{diss} (s^{-1})	Ka (M^{-1})
1E11.15	human IgG1k	HC2/KCo5 transgenic	2.7×10^{5}	4.6×10^{-5}	5.8×10^{9}
1G1.9	human IgG1k	HC2/KCo5 transgenic	9.1×10^{4}	2.2×10^{-5}	4.2×10^{9}
4D1.4	human IgG1k	HC2/KCo5 transgenic	9.8×10^{4}	4.2×10^{-5}	2.3×10^{9}
6G5.1	human IgG1k	HC2/KCo5 transgenic	1.1×10^{5}	1.0×10^{-5}	1.1×10^{10}
10C5.6	human IgG1k	HC2/KCo5 transgenic	7.4×10^{4}	1.6×10^{-5}	4.5×10^{9}
Leu3a	mouse IgG1k	Becton Dickinson	1.5×10^{5}	4.2×10^{-6}	3.7×10^{10}

<u>Mixed Lymphocyte Reaction (MLR)</u>. To compare the *in vitro* efficacy of the human monoclonal antibody 10C5.6, derived from the KCo5 transgenic mouse, to that of the mouse antibody Leu3a, an MLR assay was performed. Human PBMC

5   from 2 unrelated donors were isolated over Ficoll and CD4+ PBL from each donor were purified using a CD4 column (Human CD4 Cellect, Biotex Laboratories, Inc., Canada) according to manufacturer's directions. Inactivated stimulator cells were obtained by treating PBMC from both

10   donors with 100 mg/ml mitomycin C (Aldrich) in culture medium (RPMI 1640 with 10% heat-inactivated human AB serum (from NABI), Hepes, sodium pyruvate, glutamine, pen/strep and b-mercaptoethanol (all used at manufacturer's recommended concentrations)) for 30 min at

15   37°C followed by 3 washes with culture medium. Varying concentrations of mAbs diluted in culture medium or culture medium only were sterile filtered and added at 100 ml per well in triplicate in a 96 well round bottom plate. Fifty ml of 10^5 CD4+ PBL from one donor in culture

20   medium and 10^5 mitomycin C-treated PBMC from the other donor in 50 ml of culture medium were then added to each well. Control plates with CD4+ PBL responders alone plus mAbs were set up to control for any toxic or mitogenic effects of the mAbs. A stimulator only control and a

25   media background control were also included. After seven days in a 37°C, 5% CO_2 humidified incubator, 100 ml of supernatant from each well was removed and 20 ml of colorimetric reagent (Cell Titer 96AQ kit, Promega Corporation, Madison, WI) was added. Color was allowed

30   to develop for 4 to 6 hrs and plates were read at 490 nm. The results of this experiment, depicted in Fig. 90, show that the human IgG1k antibody 10C5.6 is at least as

effective as Leu3a at blocking the function of human PBMC
CD4 cells in this assay.

Example 40.

Binding Characteristics of Human IgGkappa Anti-CD4
monoclonal Antibodies.

This example provides the binding
characteristics of human IgGκ monoclonal antibodies
derived from hybridoma clones obtained from
HC2/KCo5/JHD/JCKD transgenic mice immunized with human
CD4. The monoclonal antibodies are shown to have high
avidity and affinity for recombinant and natural human
CD4.

Cells from 10 individual hybridoma cell lines
(1E11, 1G2, 6G5, 10C5, 1G1, 6C1, 2E4, 7G2, 1F8 and 4D1)
that secrete human IgG kappa monoclonal antibodies (mAB)
reactive with human CD4, were derived from
JHD/JCKD/HC2/KCo5 transgenic mice. The cell lines were
grown in culture, and antibody proteins were isolated
from the supernatant (Fishwild, et al. 1996, *Nature
Biotechnology* 14, 845-851, which is incorporated herein
by reference). Antibody purified by Protein A affinity
chromatography was used to measure binding constants.
The results are displayed in Tables 18 and 19.

The rate and equilibrium constants presented in
Table 18 were determined with a BIAcore (Pharmacia
Biosensor) using goat anti-human IgG (Fc-specific)
coupled to the sensor chip and flowing a saturating
concentration of mAb over followed by various
concentrations of antigen (rCD4). These constants were
derived from three experiments using purified mAbs.

Table 18. Affinity and Rate Constants.

Rate Constants (mean $\pm$ SD)

Human mAb	k_{assoc} ($M^{-1}s^{-1}$)	k_{dissoc} (s^{-1})	K_a (M^{-1})
1E11.15	1.7 ($\pm$ 0.15) x 10^5	3.5 ($\pm$ 0.09) x 10^{-3}	5.0 x 10^7
6C1.10	1.8 ($\pm$ 0.44) x 10^5	3.3 ($\pm$ 0.04) x 10^{-3}	5.4 x 10^7
1G1.9	1.2 ($\pm$ 0.18) x 10^5	9.4 ($\pm$ 0.22) x 10^{-4}	1.3 x 10^8
6G5.1	9.3 ($\pm$ 1.1) x 10^4	6.9 ($\pm$ 0.36) x 10^{-4}	1.4 x 10^8
10C5.6	9.4 ($\pm$ 0.98) x 10^4	7.1 ($\pm$ 0.36) x 10^{-4}	1.3 x 10^8
2E4.2	1.8 ($\pm$ 0.10) x 10^5	2.5 ($\pm$ 0.05) x 10^{-3}	7.1 x 10^7
4D1.4	2.5 ($\pm$ 0.55) x 10^5	3.4 ($\pm$ 0.15) x 10^{-3}	7.3 x 10^7
7G2.2	2.4 ($\pm$ 0.31) x 10^5	3.3 ($\pm$ 0.07) x 10^{-3}	7.3 x 10^7
1F8.3	1.8 ($\pm$ 0.24) x 10^5	4.3 ($\pm$ 0.14) x 10^{-3}	4.3 x 10^7
1G2.10	2.2 ($\pm$ 0.26) x 10^5	2.3 ($\pm$ 0.03) x 10^{-3}	9.8 x 10^7
chi Leu3a	1.5 ($\pm$ 0.35) x 10^5	2.3 ($\pm$ 0.12) x 10^{-4}	6.6 x 10^8

The rate and equilibrium constants presented in
Table 19 were determined with a BIAcore, using antigen
(rCD4) coupled to the sensor chip and flowing mAb over.
These constants were derived from at least three
independent experiments using purified mAbs.

Table 19. Avidity and Rate Constants

Rate Constants (mean ± SD)

Human mAb	k_{assoc} $(M^{-1}s^{-1})$	k_{dissoc} (s^{-1})	K_a (M^{-1})
1E11.15	2.8 (± 0.22) x 10^5	4.5 (± 0.43) x 10^{-5}	6.2 x 10^9
6C1.10	2.0 (± 0.25) x 10^5	4.0 (± 0.63) x 10^{-5}	5.1 x 10^9
1G1.9	9.1 (± 0.95) x 10^4	2.2 (± 0.71) x 10^{-5}	4.2 x 10^9
6G5.1	1.1 (± 0.41) x 10^5	1.0 (± 0.34) x 10^{-5}	1.1 x 10^{10}
10C5.6	7.4 (± 1.5) x 10^4	1.6 (± 0.57) x 10^{-5}	4.5 x 10^9
2E4.2	1.4 (± 0.15) x 10^5	2.2 (± 0.25) x 10^{-5}	6.3 x 10^9
4D1.4	9.8 (± 0.69) x 10^4	4.2 (± 1.3) x 10^{-5}	2.3 x 10^9
7G2.2	1.7 (± 0.20) x 10^5	5.0 (± 0.42) x 10^{-5}	3.4 x 10^9
1F8.2	1.7 (± 0.13) x 10^5	9.7 (± 1.2) x 10^{-5}	1.7 x 10^9
1G2.10	1.7 (± 0.04) x 10^5	6.3 (± 0.49) x 10^{-5}	2.7 x 10^9
chi Leu3a	4.0 (± 0.45) x 10^5	1.2 (± 0.25) x 10^{-5}	3.4 x 10^{10}
Leu3a	1.5 (± 0.30) x 10^5	4.2 (± 0.49) x 10^{-6}	3.7 x 10^{10}

Table 20 provides equilibrium constants for anti-CD4 mABs presented in the scientific literature.

Table 20. Avidity and Rate Constants Reported for Anti-CD4 monoclonal antibodies

	Rate Constants (mean ± SD)		
Human mAb	k_{assoc} (M^{-1}s^{-1})	k_{dissoc} (s^{-1})	K_a (M^{-1})
CE9.1[4]	NR*	NR	3.1×10^{10}
cMT412[1]	NR	NR	5.0×10^{9}
chi Leu3a[2]	NR	NR	1.0×10^{11}
BL4[3]	NR	NR	5.5×10^{7}
BB14[3]	NR	NR	3.3×10^{8}
cA2[5]	NR	NR	1.8×10^{9}
CDP571[6]	NR	NR	7.1×10^{9}

* NR = not reported

(1)   J. Cell. Biol. 15E:A179.

(2)   J. Immunol. 145:2839.

(3)   Clin. Immunol. Immunopath. 64:248.

(4)   Biotechnology. 10:1455.

(5)   Mol. Immunol. 30:1443.

(6)   European Patent Appl. #0626389A1.

        The avidity and affinity determinations
described above were performed with recombinant CD4
(rCD4).   To determine the avidity of the human monoclonal
antibodies for native CD4 (nCD4).   An additional binding
assay was used that does not require the antibody to be
modified.   Specifically, serial dilutions of antibody
were incubated with SupT1 cells for 6 hr on ice, washed
and detected any bound antibody with FITC-goat anti-human
Fcγ.   The Ka is determined from the concentration of
antibody that gives one-half of the maximum fluorescence
(a four parameter fit was used).   The results demonstrate

that all ten human monoclonal antibodies bind very well
to nCD4, with Ka values >10^9 M^{-1} (Table 21). Most
antibodies, including chimeric Leu3a, bound less well to
nCD4 than to rCD4. This could be due to differences in
antigen density as well as to differences between the two
antigens.

Table 21.   Avidity Constants Determined by Flow Cytometry.

Human mAb	Ka values (M-1) rCD4	nCD4*	Ratio of Ka (rCD4/nCD4)
1E11.15	6.2 x 10^9	3.3 x 10^9	1.9
6C1.10	5.1 x 10^9	3.1 x 10^9	1.6
1G1.9	4.2 x 10^9	2.3 x 10^9	1.9
6G5.1	1.1 x 10^{10}	1.9 x 10^9	5.9
10C5.6	4.5 x 10^9	1.8 x 10^9	2.5
2E4.2	6.3 x 10^9	1.1 x 10^9	5.8
4D1.4	2.3 x 10^9	2.0 x 10^9	1.2
7G2.2	3.4 x 10^9	3.3 x 10^9	1.0
1F8.2	1.7 x 10^9	3.2 x 10^9	0.5
1G2.10	2.7 x 10^9	1.9 x 10^9	1.4
chi Leu3a	3.4 x 10^{10}	5.6 x 10^9	6.1

* Human monoclonal antibodies were incubated in serial dilutions with
SupT1 cells for 6 hrs, washed twice and incubated with FITC-conjugated
goat anti-human Fcγ antisera, washed and fixed. The Ka was calculated
from the concentration of antibody yielding one-half of the maximum
fluorescence as determined from a four-parameter fit.

Example 41.

Identification of Nucleotide Sequences Encoding Human IgGkappa Anti-CD4 Antibodies.

This example demonstrates that a each of the hybridomas tested produces only one functional heavy or light chain RNA transcript, consistent with proper functioning allelic exclusion. In addition, sequence analysis of heavy and light chain CDR segments indicates that somatic mutation of the immunoglobulin transgenes has taken place.

Cells from five individual hybridoma cell lines (1E11, 1G2, 6G5, 10C5, and 4D1) that secrete human IgG kappa monoclonal antibodies reactive with human CD4, and derived from JHD/JCKD/HC2/KCo5 transgenic mice, were used to isolate RNA encoding each of the individual antibodies (Fishwild et al. 1996, *Nature Biotechnology* 14, 845-851). The RNA was used as a substrate to synthesize cDNA, which was then used to amplify human Ig gamma and kappa transcript sequences by PCR using primers specific for human VH, Vkappa, Cgamma, and Ckappa (Taylor et al. 1992, *Nucleic Acids Res.* 20, 6287-6295; Larrick, J.W., et al. (1989), *Bio/Technology.* 7. 934-938; Marks, J.D., et al. (1991). *Eur. J. Immunol.* 21. 985-991; Taylor, et al., 1994, *Int. Immunol.* 6, 579-591). The amplified Ig heavy and kappa light chain sequences were cloned into bacterial plasmids and nucleotide sequences determined. Analysis of the sequences spanning the heavy chain VDJ and light chain VJ junctions revealed in-frame heavy and light chain transcripts for each of the 5 clones, and in some cases additional out-of-frame sterile transcripts representing non-functional alleles. Consistent with proper functioning allelic exclusion, in no case was there more than one unique functional heavy or light chain transcript identified for each of the individual clones. Partial nucleotide sequences for each of the ten

functional transcripts are assigned the following
sequence I.D. No's: 1E11 gamma [Seq. I.D. No. __]; 1E11
kappa [Seq. I.D. No. __]; 1G2 gamma [Seq. I.D. No. __];
1G2 kappa [Seq. I.D. No. __]; 6G5 gamma [Seq. I.D. No.

5   __]; 6G5 kappa [Seq. I.D. No. __]; 10C5 gamma [Seq. I.D.
No. __]; 10C5 kappa [Seq. I.D. No. __]; 4D1 gamma [Seq.
I.D. No. __]; 4D1 kappa [Seq. I.D. No. __] and are
presented in Table 22. All sequences are presented in a
5' to 3' orientation.

10

       Table 22. Partial Nucleotide Sequence for
             Functional Transcripts

   1E11 gamma [Seq. I.D. No. __]

15 TGCACAAGAACATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGAT

   GGGTCCTGTCCCAGGTGCAGCTTCATCAGTGGGGCGCAGGACTGTTGAAGCCTTCGG

   AGACCCTGTCCCTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGGTTACTTCTGGA

   GCTGGATCCGCCAGCCCCCAGGGAGGGGGCTGGAGTGGATTGGGGAAATCCATCATC

   GTGGAAGCACCAACTACAACCCGTCCCTCGAGAGTCGAGTCACCCTATCAGTAGACA

20 CGTCCAAAAACCAGTTCTCCCTGAGGCTGAGTTCTGTGACCGCCGCGGACACGGCTG

   TGTATTACTGTGCGAGAGACATTACTATGGTTCGGGGAGTACCTCACTGGGGCCAGG

   GAACCCTGGTCACC

   1E11 kappa [Seq. I.D. No. __]

25 GACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTA

   CTGTCAGCAGTATGGTAGCTCACCCCTCACTTTCGGCGGAGGGACCAAGGTGGAGAT

   CAAACGAACTGTGGCGGCACCATCTGTCTTCATCTTCCC

   1G2 gamma [Seq. I.D. No. __]

30 TCCACCATCATGGGGTCAACCGCCATCCTCGCCCTCCTCCTGGCTGTTCTCCAAGGA

   GTCTGTGCCGAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAG

   TCTCTGAAGATCTCCTGTAAGGGTTCTGGATACAGCTTTACCAGTTACTGGATCGCC

TGGGTGCGCCAGATGCCCGGGAAAGGCCTGGAGTGGATGGGGATCATCGATCCTGCT

GACTCTGATACCAGATACAACCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGAC

AAGTCCATCAGTACCGCCTATTTGCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCC

ATGTATTACTGTGCGAGACCAGCGAACTGGAACTGGTACTTCGTTCTCTGGGGCCGT

5   GGCACCCTGGTCACT

1G2 kappa [Seq. I.D. No. __]

GACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTATTA

CTGTCAACAGTTTATTAGTTACCCTCAGCTCACTTTCGGCGGAGGGACCAGGGTGGA

10   GATCAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCC

6G5 gamma [Seq. I.D. No. __]

TGCACAAGAACATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGAT

GGGTCCTGTCCCAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGG

15   AGACCCTGTCCCTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGGTTACTACTGGA

GCTGGATCCGCCAGCCCCCAGGTAAGGGGCTGGAGTGGATTGGGGAAATCAATCATA.

GTGGAAGCACCAACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGTCGACA

CGTCCAAGAACCAGTTCTCCCTGAAACTGAGCTCTGTGACCGCCGCGGACACGGCTG

TGTATTACTGTGCGAGAGTAATTAATTGGTTCGACCCCTGGGGCCAGGGAACCCTGG

20   TCACC

6G5 kappa [Seq. I.D. No. __]

GACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTACTA

TTGTCAACAGGCTAATAGTTTCCCGTACACTTTTGGCCAGGGGACCAAGCTGGAGAT

25   CAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCC

10C5 gamma [Seq. I.D. No. __]

ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC

CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGGAGACCCTGTCC

30   CTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGGTTACTACTGGAGCTGGATCCGC

CAGCCCCCAGGTAAGGGGCTGGAGTGGATTGGGGAAATCAATCATAGTGGAAGCACC

AACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGTCGACACGTCCAAGAAC

CAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCGGACACGGCTGTGTATTACTGT

GCGAGAGTAATTAATTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCC

TCAG

10C5 kappa [Seq. I.D. No. __]

ATGGACATGATGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGTTCCCAGGT

TCCAGATGCGACATCCAGATGACCCAGTCTCCATCTTCCGTGTCTGCATCTGTAGGA

GACAGAGTCACCATCACTTGTCGGGCGAGTCAGGATATTAGCAGCTGGTTAGCCTGG

TATCAGCATAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTG

CAAAGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTC

ACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTACTATTGTCAACAGGCTAAT

AGTTTCCCGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAAC

4D1 gamma [Seq. I.D. No. __]

ATGGGGTCAACCGCCATCCTCGCCCTCCTCCTGGCTGTTCTCCAAGGAGTCTGTGCC

GAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAG

ATCTCCTGTAAGGGTTCTGGATACAGCTTTACCGGCTACTGGATCGGCTGGGTGCGC

CAGATGCCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGAT

ACCACATACAGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATC

AGCACCGCCTACCTGCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTAC

TGTGCGAGAGACCAACTGGGCCTCTTTGACTACTGGGGCCAGGGAACCCTGGTCACC

GTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAG

AAGCTT

4D1 kappa [Seq. I.D. No. __]

ATGGACATGGAGTTCCCCGTTCAGCTCCTGGGGCTCCTGCTGCTCTGTTTCCCAGGT

GCCAGATGTGACATCCAGATGACCCAGTCTCCATCCTCACTGTCTGCATCTGTAGGA

GACAGAGTCACCATCACTTGTCGGGCGAGTCAGGGTATTAGCAGCTGGTTAGCCTGG

TATCAGCAGAAACCAGAGAAAGCCCCTAAGTCCCTGATCTATTCTGCATCCAGTTTG

CAAAGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTC

ACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTATTACTGCCAACAGTATGAT

AGTTACCCGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGAACTGTGGCT
GCACCATCTG

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5   Analysis of these DNA sequences demonstrates

that the 5 hybridoma clones represent descendants of 4

individual primary B cells. Table 23 shows the amino

acid sequences derived for each of the ten CDR3 regions,

and the assignments for germline gene segments

10   incorporated into each of the genes encoding these

transcripts. The germline assignments are based on

published gene sequences available from the National

Center for Biotechnology Information, National Library of

Medicine, National Institutes of Health, Bethesda, Md.

15   Also see: Cook et al. 1994, Nature Genet. 7, 162-168;

Tomlinson et al. 1992, J. Mol. Biol. 227, 776-798;

Matsuda et al. 1993, Nature Genet. 3, 88-94; Schable and

Zachau, 1993, Biol. Chem. Hoppe-Seyler 374, 1001-1022;

Cox et al. 1994, Eur. J. Immunol. 24, 827-836; Ravetch et

20   al. 1981, Cell 27, 583-591; Ichihara et al. 1988, EMBO J.

7, 4141-4150; Yamada et al. 1991, J. Exp. Med. 173,

395-407; Sanz, 1991, J. Immunol. 147, 1720-1729.

Table 23. Germline V(D)J Segment Usage in Hybridoma
Transcripts.

clone	h.c. CDR3	VH	DH	JH	l.c. CDR3	Vk	Jk
1E11	DITMVRGVPH (Seq. I.D. No. __)	VH4-34	DXP'1	JH4	QQYGSSPLT (Seq. I.D. No. __)	VkA27/A11	Jk4
1G2	PANWNWYFVL (Seq. I.D. No. __)	VH5-51	DHQ52	JH2	QQFISYPQLT (Seq. I.D. No. __)	VkL18	Jk4
6G5	VINWFDP (Seq. I.D. No. __)	VH4-34	n.d.	JH5	QQANSFPYT (Seq. I.D. No. __)	VkL19	Jk2
10C5	VINWFDP	VH4-34	n.d.	JH5	QQANSFPYT	VkL19	Jk2
4D1	DQLGLFDY (Seq. I.D. No. __)	VH5-51	DHQ52	JH4	QQYDSYPYT (Seq. I.D. No. __)	VkL15	Jk2

n.d. could not be determined from nucleotide sequence.

Example 42.

Construction of Minigenes for Expression of Human IgGkappa AntiCD4 Antibodies in Transfected Cell lines.

This example demonstrates the process of making a wholly artificial gene that encodes an immunoglobulin polypeptide (i.e., an immunoglobulin heavy chain or light chain). Plasmids were constructed so that PCR amplified V heavy and V light chain cDNA sequences could be used to reconstruct complete heavy and light chain minigenes.

The kappa light chain plasmid, pCK7-96, includes the kappa constant region and polyadenylation site [Seq. ID No. __], such that kappa sequences amplified with 5' primers that include HindIII sites
5   upstream of the initiator methionine can be digested with HindIII and BbsI, and cloned into pCK7-96 digested with HindIII and BbsI to reconstruct a complete light chain coding sequence together with a polyadenylation site. This cassette can be isolated as a HindIII/NotI fragment
10   and ligated to transcription promoter sequences to create a functional minigene for transfection into cells.

The gamma1 heavy chain plasmid, pCG7-96, includes the human gamma1 constant region and polyadenylation site [Seq. ID No. __], such that gamma
15   sequences amplified with 5' primers that include HindIII sites upstream of the initiator methionine can be digested with HindIII and AgeI, and cloned into pCG7-96 digested with HindIII and AgeI to reconstruct a complete gamma1 heavy chain coding sequence together with a
20   polyadenylation site. This cassette can be isolated as a HindIII/SalI fragment and ligated to transcription promoter sequences to create a functional minigene for transfection into cells.

The following example demonstrates how
25   nucleotide sequence data from hybridomas can be used to reconstruct functional Ig heavy and light chain minigenes. The nucleotide sequences of heavy and light chain transcripts from hybridomas 6G5 and 10C5 were used to design an overlapping set of synthetic
30   oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and kappa light chain

sequences (designated HC6G5 [Seq. I.D. No. __] and LC6G5 [Seq. I.D. No. __] differed from the natural sequences in three ways: strings of repeated nucleotide bases were interrupted to facilitate oligonucleotide synthesis and PCR amplification; optimal translation initiation sites were incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266, 19867-19870); and, HindIII sites were engineered upstream of the translation initiation sites.

A. <u>Synthetic kappa light chain.</u>

Light chain PCR reaction 1.

The following oligonucleotides were pooled: o-548 [Seq. I.D. No. __], o-549 [Seq. I.D. No. __], o-550 [Seq. I.D. No. __], o-551 [Seq. I.D. No. __], o-552 [Seq. I.D. No. __], o-563 [Seq. I.D. No. __], o-564 [Seq. I.D. No. __], o-565 [Seq. I.D. No. __], o-566 [Seq. I.D. No. __], o-567 [Seq. I.D. No. __], and amplified with the following 2 primers: o-527 [Seq. I.D. No. __] and o-562 [Seq. I.D. No. __].

Light chain PCR reaction 2.

The following oligonucleotides were pooled: o-553 [Seq. I.D. No. __], o-554 [Seq. I.D. No. __], o-555 [Seq. I.D. No. __], o-556 [Seq. I.D. No. __], o-557 [Seq. I.D. No. __], o-558 [Seq. I.D. No. __], o-559 [Seq. I.D. No. __], o-560 [Seq. I.D. No. __], o-561 [Seq. I.D. No. __], o-562 [Seq. I.D. No. __], and amplified with the following 2 primers: o-552 [Seq. I.D. No. __] and o-493 [Seq. I.D. No. __].

Light chain PCR reaction 3.

The products of light chain PCR reactions 1 and
2 were then combined and amplified with the following two
primers: o-493 [Seq. I.D. No. __] and o-527 [Seq. I.D.
No. __].

5   The product of light chain PCR reaction 3 was
then digested with HindIII and BbsI and cloned into
HindIII/BbsI digested pCK7-96 [Seq. I.D. No. __] to
generate pLC6G5 [Seq. I.D. No. __].

10

B. <u>Synthetic gamma heavy chain.</u>
Heavy chain PCR reaction 1.

          The following oligonucleotides were pooled: o-
528 [Seq. I.D. No. __], o-529 [Seq. I.D. No. __], o-530
15   [Seq. I.D. No. __], o-531 [Seq. I.D. No. __], o-532 [Seq.
I.D. No. __], o-543 [Seq. I.D. No. __], o-544 [Seq. I.D.
No. __], o-545 [Seq. I.D. No. __], o-546 [Seq. I.D. No.
__], o-547 [Seq. I.D. No. __], and amplified with the
following 2 primers: o-496 [Seq. I.D. No. __] and o-542
20   [Seq. I.D. No. __].

Heavy chain PCR reaction 2.

          The following oligonucleotides were pooled: o-
533 [Seq. I.D. No. __], o-534 [Seq. I.D. No. __], o-535
25   [Seq. I.D. No. __], o-536 [Seq. I.D. No. __], o-537 [Seq.
I.D. No. __], o-538 [Seq. I.D. No. __], o-539 [Seq. I.D.
No. __], o-540 [Seq. I.D. No. __], o-541 [Seq. I.D. No.
__], o-542 [Seq. I.D. No. __], together with the isolated
439 bp BbsI fragment of pCG7-96 [Seq. I.D. No. __] and
30   amplified with the following 2 primers: o-490 [Seq. I.D.
No. __] and o-520 [Seq. I.D. No. __].

Heavy chain PCR reaction 3.

The products of heavy chain reactions 1 and 2
were then combined and amplified with the following two
primers: o-520 [Seq. I.D. No. __] and o-521 [Seq. I.D.
No. __].

The product of heavy chain reaction 3 was then
digested with HindIII and AgeI and cloned into
HindIII/AgeI digested pCG7-96 [Seq. I.D. No. __] to
generate pHC6G5 [Seq. I.D. No. __].

Table 24.   Primers, Vectors and Products
Used in Minigene Construction

pCK7-96 [Seq. I.D. No. __]
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGC
TCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT
GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC
GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTC
AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAG
CCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACAC
GACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTA
GGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACA
GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGC
TCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAG
CAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGG
TCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCA
AAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAA
AGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCT
ATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAG

ATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGA
GACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCC
GAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATT
GCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGT
TCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGC
TCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATG
GTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT
GTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGT
TGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAA
GTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTG
TTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTT
ACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAG
GGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTAT
TGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAG
AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC
TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCC
TTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCG
GAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGC
GCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAG
ATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGA
AAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGA
TCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGG
CGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC
AGTGCCAAGCTAGCGGCCGCGGTCCAACCACCAATCTCAAAGCTTGGTACCCGGGAG
CCTGTTATCCCAGCACAGTCCTGGAAGAGGCACAGGGGAAATAAAAGCGGACGGAGG
CTTTCCTTGACTCAGCCGCTGCCTGGTCTTCTTCAGACCTGTTCTGAATTCTAAACT
CTGAGGGGGTCGGATGACGTGGCCATTCTTTGCCTAAAGCATTGAGTTTACTGCAAG
GTCAGAAAAGCATGCAAAGCCCTCAGAATGGCTGCAAAGAGCTCCAACAAAACAATT
TAGAACTTTATTAAGGAATAGGGGGAAGCTAGGAAGAAACTCAAAACATCAAGATTT
TAAATACGCTTCTTGGTCTCCTTGCTATAATTATCTGGGATAAGCATGCTGTTTTCT
GTCTGTCCCTAACATGCCCTGTGATTATCCGCAAACAACACACCCAAGGGCAGAACT

TTGTTACTTAAACACCATCCTGTTTGCTTCTTTCCTCAGGAACTGTGGCTGCACCAT

CTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTG

TGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATA

ACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACA

5 GCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACA

AAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCT

TCAACAGGGGAGAGTGTTAGAGGGAGAAGTGCCCCCACCTGCTCCTCAGTTCCAGCC

TGACCCCCTCCCATCCTTTGGCCTCTGACCCTTTTTCCACAGGGGACCTACCCCTAT

TGCGGTCCTCCAGCTCATCTTTCACCTCACCCCCCTCCTCCTCCTTGGCTTTAATTA

10 TGCTAATGTTGGAGGAGAATGAATAAATAAAGTGAATCTTTGCACCTGTGGTTTCTC

TCTTTCCTCAATTTAATAATTATTATCTGTTGTTTACCAACTACTCAATTTCTCTTA

TAAGGGACTAAATATGTAGTCATCCTAAGGCGCATAACCATTTATAAAAATCATCCT

TCATTCTATTTTACCCTATCATCCTCTGCAAGACAGTCCTCCCTCAAACCCACAAGC

CTTCTGTCCTCACAGTCCCCTGGGCCATGGATCCTCACATCCCAATCCGCGGCCGCA

15 ATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC

CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA

GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGT

CGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG

GGCGC

20

pCG7-96 [Seq. I.D. No. __]

GAACTCGAGCAGCTGAAGCTTTCTGGGGCAGGCCAGGCCTGACCTTGGCTTTGGGGC

AGGGAGGGGGCTAAGGTGAGGCAGGTGGCGCCAGCCAGGTGCACACCCAATGCCCAT

GAGCCCAGACACTGGACGCTGAACCTCGCGGACAGTTAAGAACCCAGGGGCCTCTGC

25 GCCCTGGGCCCAGCTCTGTCCCACACCGCGGTCACATGGCACCACCTCTCTTGCAGC

CTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGG

GGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGT

GTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACA

GTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTT

30 GGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGA

CAAGAAAGTTGGTGAGAGGCCAGCACAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGC

TCAGCGCTCCTGCCTGGACGCATCCCGGCTATGCAGCCCCAGTCCAGGGCAGCAAGG

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CAGGCCCCGTCTGCCTCTTCACCCGGAGGCCTCTGCCCGCCCCACTCATGCTCAGGG
AGAGGGTCTTCTGGCTTTTTCCCCAGGCTCTGGGCAGGCACAGGCTAGGTGCCCCTA
ACCCAGGCCCTGCACACAAAGGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATA
TCCGGGAGGACCCTGCCCCTGACCTAAGCCCACCCCAAAGGCCAAACTCTCCACTCC
CTCAGCTCGGACACCTTCTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTG
CAGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGGTAAGCCAG
CCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCC
AGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCTCTTCCTCAGCACCT
GAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTC
ATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGAC
CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACA
AAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTC
CTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCC
CTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGTGGGACCCGTGGGGTG
CGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCCTCTGCCCTGAGAGTGACCGC
TGTACCAACCTCTGTCCCTACAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCC
CCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG
CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA
CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAA
GCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGAT
GCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAA
ATGAGTGCGACGGCCGGCAAGCCCCCGCTCCCCGGGCTCTCGCGGTCGCACGAGGAT
GCTTGGCACGTACCCCCTGTACATACTTCCCGGGCGCCCAGCATGGAAATAAAGCAC
CCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGATGGTTCTTTCCACGGGTCAGGCCG
AGTCTGAGGCCTGAGTGGCATGAGGGAGGCAGAGCGGGTCCCACTGTCCCCACACTG
GCCCAGGCTGTGCAGGTGTGCCTGGGCCCCCTAGGGTGGGGCTCAGCCAGGGGCTGC
CCTCGGCAGGGTGGGGGATTTGCCAGCGTGGCCCTCCCTCCAGCAGCACCTGCCCTG
GGCTGGGCCACGGGAAGCCCTAGGAGCCCCTGGGGACAGACACACAGCCCCTGCCTC
TGTAGGAGACTGTCCTGTTCTGTGAGCGCCCCTGTCCTCCCGACCTCCATGCCCACT
CGGGGGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCA
TCGATGATATCAGATCTGCCGGTCTCCCTATAGTGAGTCGTATTAATTTCGATAAGC
CAGGTTAACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATT
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GGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGG

CGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGAT

AACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG

GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAAT

5

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CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGG

TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC

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O-549 [Seq. I.D. No. __]

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O-550 [Seq. I.D. No. __]

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O-551 [Seq. I.D. No. __]

20   AGTCAGGATATTAGCAGCTGGTTAGCCTGGTATCAGCATAAACC

O-552 [Seq. I.D. No. __]

AGGTAAAGCACCTAAGCTCCTGATCTATGCTGCATCCAGTTTGC

25   O-563 [Seq. I.D. No. __]

AGGAGCTTAGGTGCTTTACCTGGTTTATGCTGATACCAGGCTAA

O-564 [Seq. I.D. No. __]

CCAGCTGCTAATATCCTGACTCGCCCGACAAGTGATGGTGACTC

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O-565 [Seq. I.D. No. __]

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O-566 [Seq. I.D. No. __]

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O-567 [Seq. I.D. No. __]

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O-527 [Seq. I.D. No. __]

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O-562 [Seq. I.D. No. __]

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O-553 [Seq. I.D. No. __]

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O-554 [Seq. I.D. No. __]

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O-555 [Seq. I.D. No. __]

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O-556 [Seq. I.D. No. __]

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O-557 [Seq. I.D. No. __]

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O-558 [Seq. I.D. No. __]

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O-559 [Seq. I.D. No. __]

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O-560 [Seq. I.D. No. __]

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O-561 [Seq. I.D. No. __]

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pLC6G5 [Seq. I.D. No. __]

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TCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT

GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC

GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTC

AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAG

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O-543 [Seq. I.D. No. __]

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O-533 [Seq. I.D. No. __]

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O-536 [Seq. I.D. No. __]

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O-540 [Seq. I.D. No. __]

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O-538 [Seq. I.D. No. __]

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O-490 [Seq. I.D. No. __]

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O-520 [Seq. I.D. No. __]

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O-521 [Seq. I.D. No. __]

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HC6G5 [Seq. I.D. No. __]

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CCCGAACCGGT

LC6G5 [Seq. I.D. No. __]

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GCTAATAGTTTCCCGTACACTTTTGGTCAGGGAACCAAGCTGGAGATCAAACGAACT

GTGGCTGCACCATCTGTCTTC

5   Example 43.
            Binding of Human Anti-CD4 Monoclonal Antibodies to
                     Non-Human Primate Lymphocytes.

            It is desirable to be able perform preclinical

toxicology and pharmacokinetic studies of human anti-CD4

10   monoclonal antibodies in animal models. It is further

desirable for some purposes that the animal be a

non-human primate that expresses CD4 comprising a

cross-reactive epitope with human CD4 such that it is

recognized by the monoclonal antibody. Three different

15   non-human primate species, chimpanzee, rhesus, and

cynomolgus monkeys, were tested for cross-reactive CD4

epitopes with the 5 different human anti-CD4 monoclonal

antibodies from hybridomas 1E11, 1G2, 6G5, 10C5, and 4D1.

Peripheral blood lymphocytes were isolated from whole

20   blood of chimpanzee, rhesus, and cynomolgus monkeys. The

isolated cells were double stained with human antibody

from each of these 5 hybridomas (detected with

FITC-anti-human IgG) and PE-anti-CD8 or PE-anti-CD4. The

stained cells were then analyzed by flow cytometry to

25   determine if each of the human monoclonal antibodies

bound to endogenous CD4 on the surface of lymphocytes

from each of these three non-human primates. Four of the

five antibodies, 1E11, 6G5, 10C5, and 4D1, were found to

bind to chimpanzee CD4 cells. Additionally, four of the

30   five antibodies, 6G5, 1G2, 10C5, and 4D1, were found to

bind to both rhesus and cynomolgus CD4 cells. Thus,

three of five antibodies, 6G5, 10C5, and 4D1, bind to CD4

cells in each of the three non-human primate species tested.

Example 44

Lack of Correlation between Modulation and Non-Depletion

There are no known *in vitro* assays that can reliably predict whether a monoclonal antibody (mAb) will be nondepleting or immunosuppressive in patients. However, a correlation has been observed between the ability of three different mAbs to deplete (or not deplete) in humans and nonhuman primates such as chimpanzees and cynomolgus monkeys (See, e.g., M. Jonker *et al., Clin. Exp. Immunol.,* 93:301-307 (1993); and J.A. Powelson *et al., Transplantation,* 57:788-793 (1994)). Therefore a study was performed using human mAbs in nonhuman primates.

Chimpanzees were used in this study, because one of the anti-CD4 mAbs, 1E11, recognizes CD-4 only in chimpanzees and not in Rhesus or cynomolgus monkeys. A second mAb, 6G5, recognizes CD4 in chimpanzees, Rhesus and cynomolgus monkeys. A third mAb, 1G2, does not recognize CD4 in chimpanzees, but does in Rhesus and cynomolgus monkeys. That mAb has already been shown to be nondepleting *in vivo* in cynomolgus monkeys.

In addition to examining the effect of human mAbs on CD4+ T cell numbers in peripheral blood, the effect of the mAb administration on *in vivo* T cell function was also evaluated. The most accepted manner to do this is to use animals that have been presensitized to an antigen such as tuberculin or tetanus toxoid and who will mount a hypersensitivity reaction in the skin.

Three male chimpanzees were enrolled in this study. Baseline whole blood samples were obtained on days -7, -3 and 1. After the blood draw on day 1, one chimpanzee each was intravenously infused with one of the two human mAbs (1E11 or 6G5) at 2 mg/kg. The third chimpanzee received an equal volume/kg of buffer only. Blood was drawn at 30 mins, 2 hrs, 8 hrs, 24 hrs and 48 hrs post-infusion. On day 2, a skin reactivity test was performed.

Results shown in Table 25 below clearly demonstrate that 1E11 caused transient depletion of peripheral lymphocytes, with most CD4+ T cells being depleted. Even though 6G5 did not cause lymphocyte or CD4+ T cell depletion, both mAbs were able to inhibit a hypersensitivity response to tetanus toxoid, compared to the control chimpanzee. Thus, both human mAbs appear to be immunosuppressive *in vivo*, and this immunosuppression does not necessarily require T cell depletion.

Table 25. Effect of Human mAbs on Peripheral Chimpanzee Lymphocytes

Peripheral Lymphocytes (million/ml)

Study Day	1E11	6G5	Control
-7	4.2	6.4	4.2
-4	4.0	9.9	4.4
1, pre-infusion	4.8	5.7	5.8
1, 30 min post	1.6	6.0	4.0
1, 2 hr post	1.0	6.7	5.2
1, 6 hr post	1.5	8.0	4.2
2	3.5	9.6	5.7
3	3.9	9.7	5.9

Whole blood samples from the chimpanzees were collected on study days 5, 8, 15 and 29. PBMC from all blood samples were isolated and examined by flow cytometry to determine the percent of CD4 T, CD45RA/CD4 T (naive), CD45RO/CD4 T (memory), CD8 T and CD19 B cells present in PBMC; the density of CD4 molecules per cell; and whether the human mAbs had bound to those cells. The cells were prepared for flow cytometry as described (Fishwild et al., 1996, *Nature Biotechnology* 14:845-851). Complete blood counts were performed on a sample of whole blood to quantitate lymphocytes. The total number of cells for any given subset was then derived by multiplying the percent of such cells by the total number of lymphocytes.

The CD4^{+} cells were determined from positive staining of PBMC with PE-OKT4 (Fisher) and from negative staining with FITC-CD8 (CalTag). The % CD3^{+},CD8^{-},CD45RA^{+} (naive CD4^{+} T) cells and the % CD3^{+}, CD8^{-}, CD45RO^{+} (memory CD4^{+} T) cells were determined from PBMC stained with TriColor-CD8 (CalTag), FITC-Leu4a (Becton-Dickinson) and

PE-CD45RA (CalTag) or with TriColor-CD8 , FITC-Leu4a and

PE-CD45RO (CalTag), respectively. The total number of

CD4⁺ cells (top left) and the ratio of naive to memory CD4

cells (top right) were then calculated. The amount of

5   CD4 (bottom left) was determined from the mean channel

fluorescence of PE-OKT4⁺ lymphocytes. The amount of

human mAb bound to CD4⁺ (PE-BF5⁺, SeroTec) cells (bottom

right) was determined from the mean channel fluorescence

(MCF) of FITC-goat anti-human Fcγ+ cells. Results

10   obtained for gated lymphocytes only are shown here.

As shown in Figure 91, only 1E11 and not 6G5

depleted CD4⁺ T cells in circulation, even though both

bound equally well to CD4⁺ T cells. (*Depletion* refers to

loss or decrease in numbers of specific immune cells from

15   circulation, from lymphoid tissues, or from both.) As

expected, 1E11 strongly and 6G5 weakly modulated CD4

antigen on T cells *in vivo* (Figure 91). The slightly

lower binding of 1E11 to CD4⁺ cells as compared to 6G5

seen from 30 min through 2 days is probably a consequence

20   of the greater CD4 modulation observed with 1E11.

(*Modulation* refers to loss of antigen from the surface of

a cell, or a decrease in surface antigen density. The

loss can occur as a result of shedding or internalization

of the antigen, usually in the form of antigen-antibody

25   complexes.) Memory and naive CD4⁺ cells were equally

affected by 1E11. Not unexpectedly, 1E11 depleted

monocytes (which are CD4⁺) until day 5, whereas 6G5 did

not.

There was transient and nonspecific depletion

30   of CD8⁺ cells only in the 1E11 animal (Figure 92), whereas

CD19⁺ B cells were not affected in any of the chimpanzees.

The total number of CD8⁺,CD3⁺ (T suppressor) cells (left)

and the total number of CD19^{+} cells (right) were
determined from PBMC co-stained with PE-CD8 and
FITC-Leu4a or stained with FITC-CD19 (CalTag),
respectively.

In summary, 1E11 which induced extensive CD4
modulation both *in vitro* and *in vivo*, unexpectedly
depleted CD4^{+} T cells. 6G5 which induces moderate CD4
modulation both *in vitro* and *in vivo*, unexpectedly did
not deplete CD4^{+} T cells. Thus, the seeming correlation
between modulation and nondepletion that had been
observed for other anti-CD4 mAbs has been disproven.

Example 45

Prevention of T-helper Dependent Immune Response *In Vivo*

The animals described in Example 44 were
immunized by injection of 1.5 ml of tetanus toxoid (TT,
Fort Dodge Animal Health Care, Fort Dodge, IA)
intramuscularly in the thigh on day -21. The animals
received 0.1 ml of a 10% TT solution or saline alone
intradermally on the back on days 2 and 29. The site was
examined 24 hrs later. Reactions were scored as 0 if
there was no change at the site of injection, 1 if the
injection site was reddened, 2 if the injection site was
reddened and raised, and 3 if the injection site was
reddened, raised and firm. On day 2, in chimpanzees
receiving either of the two human mAbs, 1E11 or 6G5,
there was no injection site reaction to TT while the
chimpanzee receiving the PBS injection had a reddened
injection site. On day 29, all chimpanzees had reddened
injection site reactions for TT. There was no reaction
at the site where saline had been injected on either day.

Thus, while the human mAbs were present (on day 2), they apparently were able to prevent a T-helper dependent immune response *in vivo*.

Example 46

Depletion Studies in the Cynomolgus Monkey

Depletion studies were also carried out in the cynomolgus monkey. A baseline whole blood sample was obtained on day 0 from four cynomolgus monkeys. After this blood draw, one cynomolgus monkey each was intravenously infused with one of the three mAbs (1E11, 6G5 or 1G2) at 2 mg/kg. The fourth cynomolgus monkey received the same volume/kg of PBS only. Blood was drawn at 2 hrs and 8 hrs post-infusion on day 0. Blood was drawn daily (approx. every 24 hrs after the infusion) on days 1, 2, 4, 7, 11, 18 and 32. PBMC from whole blood were isolated over Ficoll following standard procedures. Aliquots of the PBMC were then stained as in Example 44 with various fluorochrome monoclonal antibody conjugates to monitor CD4$^+$ T cells and CD8$^+$ T cells, and human monoclonal antibody bound to CD4$^+$ T cells.

As described in Example 43, in this species, 1E11 mAb does not recognize CD4, whereas both 6G5 and 1G2 mAbs do. Thus, 1E11 serves as a negative control human mAb in the cynomolgus study.

As shown in Figure 93, none of the antibodies depleted CD4$^+$ T cells including the poorly modulating 6G5 mAb. Nor was there any effect on CD8$^+$ T cells. Both 6G5 and 1G2 bound to CD4$^+$ T cells, but only 1G2 induced CD4 modulation *in vivo*. Moreover, although both 1G2 and 1E11 extensively modulated CD4 *in vivo*, one mAb was depleting

and one was not. Therefore, there appears to be no
correlation between modulation and depletion. In Figure
93, the percent of CD4⁺ cells (top left) and the percent
of CD8⁺ cells (top right) are shown. The CD4⁺ cells were
determined from positive staining of PBMC with PE-OKT4
(Ortho) and from negative staining with FITC-CD8
(Becton-Dickinson). The amount of CD4 (bottom left) was
determined from the MCF of PE-OKT4⁺ lymphocytes. The
amount of human mAb bound to CD4⁺ (PE-OKT4⁺) cells (bottom
right) was determined from the MCF of FITC-goat
anti-human FcY⁺ cells. Result obtained for gated
lymphocytes only are shown.

Example 47

Lymph Node Lymphocytes

To determine whether the human mAbs were able
to exit the vasculature and appear in other peripheral
organs, as part of the previously described chimpanzee
study, inguinal lymph nodes were examined on days -7, 2
and 29. Biopsies were performed and single cell
suspensions in medium were prepared. The percent of CD4,
CD8 and CD3 cells present in the lymph node mononuclear
cells was determined, and, using the methods described in
Examples 43-45, it was determined whether the human mAbs
had bound to the lymph node mononuclear cells.

As shown in Table 26 and Figure 94, both 1E11
and 6G5 slightly depleted CD4⁺ T cells in lymph nodes on
day 2. This effect was reversed on day 29 for 6G5 and
became even more apparent for 1E11. There was a
corresponding increase in CD8⁺ T cells on all days when
CD4⁺ T cells were depleted. Part, but not all, of the

explanation for decreased CD4$^+$ T cells on day 29 in
chimpanzee A008 may be due to a lowered percent of CD3$^+$
cells (and concomitantly increased percent of B cells).
Both 1E11 and 6G5 bound to CD4$^+$ T cells in lymph nodes.
Thus, these two mAbs were able to exit the blood and
enter lymph tissue. Whether they did so as soluble
antibodies or attached to CD4 cells can not be determined
from these data. 1E11 modulated CD4 antigen in lymph
nodes, whereas 6G5 minimally modulated CD4, if at all.
This effect was observed only on day 2.

In Figure 94, the % CD4$^+$ T cells (top left) and
on the % CD8$^+$,CD3$^+$ T cells (top right) were determined
from positive staining of lymph node lymphocytes with
PE-OKT4 and from negative staining with FITC-CD8 or from
lymphocytes co-stained with PE-CD8 and FITC-Leu4a. The
amount of CD4 (bottom left) as determined from the MCF of
PE-OKT4$^+$ lymphocytes is shown. The amount of human
monoclonal antibody bound to CD4$^+$ (PE-BF5$^+$) cells (bottom
right) was determined from the MCF of FITC-goat
anti-human Fcγ$^+$ cells.

Table 26.

Summary of Flow Cytometry Studies on
Lymph Node Lymphocytes

Cell Type	Chimpanzee Number (Article Injected)		
	A008 (1E11)	A010 (6G5)	A017 (PBS)
%CD4$^+$	Decr on d2, d29	Decr on d2	
%CD8$^+$	Incr on d2, d29	Incr on d2	
%CD3$^+$	Decr on d29		
%CD19$^+$	Incr on d29		
MCF CD4	Decr on d2		
MCF HuMAb	Incr on d2	Incr on d2	

Blanks mean that there was no significant change in the parameter being
examined.
Decr = decrease   Incr = increase

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In summary, it is apparent from these data that human mAbs could migrate into lymphoid tissue. These mAbs were cleared from the lymph nodes between days 2 and 29. When present in the lymph node, 1E11 induced CD4 modulation, although to a lesser extent than in peripheral blood on the same study day. One of these antibodies, 1E11, depleted CD4+ T cells from lymph nodes on day 2 and 29. Thus, 1E11 appears to be able to deplete CD4+ T cells from peripheral organs for a longer period of time than it does from peripheral blood.

Example 48

Half life of Human Monoclonal Antibodies in Nonhuman Primates.

As part of the cynomolgus monkey study (described in Example 46), plasma samples were obtained at various time points and assayed for the presence of human mAb. The standard quantitative rCD4 ELISA (Lonberg et al., 1994, Nature 368:856-9) was used, except that the cynomolgus plasma was diluted 1:1000 and 1:10000 prior to assay and the mAb standards were diluted in 1:1000 and 1:10000 normal cynomolgus plasma in diluent buffer instead of the diluent buffer alone.

As shown in Figure 95, the serum half-life of 1E11, the human mAb which does not recognize cynomolgus CD4, was 10 days. The serum half-life of the human mAbs which do recognize cynomolgus CD4, 6G5 and 1G2, were 39 and 14 hrs, respectively. Thus, it would appear that the presence of an antigen sink can significantly decrease the half-life of human mAbs and that antigen turnover (as occurs with CD4 modulation) can further decrease serum-half life. Thus, the most desirable mAb for

chronic clinical use would be a non-depleting, non-modulating mAb such as 6G5.

Example 49

Effect on Response to Tetanus Toxoid

It has been shown above that all human IgGκ anti-CD4 mAbs that recognized the membrane distal domains of CD4 were able to inhibit responses to alloantigen (cell surface expressed MHC class II molecules) *in vitro*, as in a mixed lymphocyte reaction (MLR). The ability of these human anti-CD4 mAbs to inhibit human cell responses to a soluble foreign antigen, tetanus toxoid (TT), was then determined *in vitro*. PBMC from human donors were prescreened for reactivity to TT, and the reactive PBMC stored at -80°C and subsequently assayed for antibody inhibition using freshly thawed PBMC from those responsive donors. Serial dilutions of human monoclonal antibodies were added to 1×10^5 PBMC per well in medium followed by 5 LF/ml of TT (Wyeth-Ayerst). Cells were cultured for a total of 7 days and ^{3}H-thymidine was added 16 hr prior to harvest.

As shown in Figure 96, all three anti-CD4 mAbs tested inhibited responses to TT. 1E11 antibody was much more potent than either 6G5 or 1G2. The negative control human mAb, 4E4, had no effect.

Example 50

Production of human anti-IL8 mAbs

Recombinant human IL8 (rhIL8) was expressed in *E. coli* using the pET system (Novagen) and purified to N-

terminal sequence homogeneity using heparin affinity
chromotography (hi-trap, Pharmacia) followed by size
exclusion chromotography (Superdex 75, Pharmacia) and C18
reverse phase HPLC (ultrasphere ODS, Beckman). HuMAb

5   transgenic mice were immunized with 20 ug recombinant
human IL8 in complete Fruend's adjuvant, then with 4-5
weekly injections of 5 ug rhIL8 in incomplete Fruend's
adjuvant.   In the first and second round of
immunizations, transgenic mice from three different

10   strains were used (five HCo7/KCo4 mice, nine HC2/KCo5
mice, and three HC2/HCo7/KCo4/KCo5 mice; all were
JhD/JkD).   In the third round of immunizations,
transgenic mice from two different strains were used
(four HC2/KCo5 mice, and six HCo7/KCo5 mice; all were

15   CmD/JkD).   Serum was removed prior to each immunization
and tested for the presence of human IgG and IgM
antibodies by ELISA.   Briefly, wells of microtiter plates
were coated with 0.2 ug/ml rhIL8 in carbonate buffer
overnight at 4°C, blocked with 5% chick serum in PBS for 2

20   hrs at 37°C, incubated with goat anti-human IgG or IgM for
1 hr at 37°C, and finally incubated with ABTS substrate
for 1 hr at 25°C.   Plates were washed extensively between
each step.

       All transgenic mice from all strains responded
25   readily to immunization with rhIL8, with human IgM
antibodies detected prior to human IgG antibodies in
serum.   Human IgG responses peaked around week 4 and did
not increase with further immunizations as shown in
Figure 97.   In general, mice containing the HCo7

30   transgene responded slightly better than those containing
only the HC2 transgene.   This may be related to the
particular V regions present in these two transgenes (of

the four Vh regions, only one is common to both

transgenes) or to the site of intergration or to some.

other as yet undetermined factor. In any case, the

difference in responsiveness was small.

5   Spleens from four mice (two were from

HC2/HCo7/KCo4/KCo5/JhD/JkD mice and two were from

HCo7/KCo5/CmD/JkD mice) were removed and processed for

fusion by as described (Fishwild et al. 1996, *Nature*

Biotechnology 14:845-851). These mice were chosen

10   because they had the highest titers of human IgG anti-IL8

antibodies in serum. A total of 29 hybridomas secreting

human IgGk anti-rhIL8 mAbs were detected in parental

wells. Of the 21 parental hybridomas attempted, 18 of

these hybridomas were successfully subcloned. There was

15   no significant difference between these two transgenic

strains with respect to either the total number of

parental hybridomas detected or to their subcloning

efficiency.

20

Example 51

Characterization of human IgGk anti-IL8 mAbs

The first 14 subcloned hybridomas obtained and their

secreted mAbs were extensively characterized (all obtained

25 from HC2/HCo7/KCo4/KCo5/JhD/JkD mice). The other 4 subcloned

hybridomas have been partially characterized.

The amount of mAb secreted by each hybridoma was

determined by a quantitative ELISA as previously described

(Lonberg et al., 1994, *Nature* 368:856-9). The on- and

30 off-rates for human mAbs were also determined as described

(Lonberg et al., 1994, *Nature* 368:856-9), except that rhIL8

was coupled to the BIAcore sensor chip instead of rCD4.

As shown in Table 27, most of these hybridomas secreted very high amounts of human antibody (>10 μg/ml), ranging from 0.5 to 32 μg/ml. Most of these mAbs had very high binding avidities, with Ka values >10^9 M^{-1}. There was

5   quite a large range of on-rates (k_{assoc}) and off-rates (k_{dissoc}). Moreover, many of the on- and off-rates are unique, suggesting that among these 14 mAbs, there may be at least 10 distinct mAbs (i.e., not derived from the same parental splenic B cell).

10

Table 27.

mAb Secretion, Avidity and Rate Constants

Human mAb (mouse #)	mAb Conc (mg/ml)	Rate Constants		Ka (M⁻¹)
		k_{assoc} (M⁻¹s⁻¹)	k_{dissoc} (s⁻¹)	
1F8.19	17	2.7×10^4	1.5×10^{-5}	1.8×10^9
(14476)				
2D11.8	1.8	4.6×10^5	8.1×10^{-5}	5.6×10^9
2D11.6	1.2	5.3×10^5	2.2×10^{-5}	2.3×10^{10}
2F9.5	1.2	4.4×10^5	3.0×10^{-5}	1.5×10^{10}
2F9.4	1.0	4.4×10^5	1.4×10^{-5}	3.2×10^{10}
2G1.8	1.3	4.9×10^5	5.7×10^{-5}	8.7×10^9
3E5.4	26	1.3×10^5	4.3×10^{-5}	3.1×10^9
5E7.4	0.5	nd	nd	
5F10.6	11	4.6×10^4	5.2×10^{-6}	8.8×10^9
5H8.8	16	7.7×10^4	6.3×10^{-5}	1.2×10^9
2C6.1	32	9.9×10^4	2.7×10^{-5}	3.6×10^9
(14477)				
2D6.3	6.3	9.7×10^4	4.6×10^{-5}	2.1×10^9
3A1.7	20	1.2×10^5	1.5×10^{-4}	8.2×10^8
4D4.8	14	6.3×10^4	6.7×10^{-5}	9.4×10^8
7C5.7	20	4.0×10^4	1.5×10^{-4}	2.6×10^8
10A6.9	6.2	1.0×10^5	7.0×10^{-5}	1.5×10^9

The rate and equilibrium constants for monoclonal antibodies (mAb) were
determined with a BIAcore, using antigen (rhIL8) coupled to the sensor chip and
flowing mAb over. These constants were derived from one experiment using mAbs
in spent tissue culture medium.
nd = not determined

The mAbs were then tested for specificity. Various

CXC chemokines (IL8, IP10, Nap2, ENA78, GROα, GROβ and GROγ)

were absorbed to ELISA plates and the binding of the human

mAbs to those chemokines detected by an enzyme conjugated

anti-human Ig antiserum as described in Example 49 for the IL8

ELISA. Specificity as well as the ability to neutralize IL8

was determined by examining whether the human mAbs could

inhibit 1) the binding of radiolabeled IL8 to IL8RA-expressing

transfectants, 2) the binding of radiolabeled IL8 to

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neutrophils, 3) the binding of radiolabeled GROa to IL8RB-expressing transfectants and 4) the effect on IL8-induced Ca^{++} flux in neutrophils.

Stable IL8RA-expressing transfectants were created

5 using the murine pre-B lymphoma cell line (L1-2) as described (Campbell *et al.*,1996, *J. Cell Biol.* 134:255-66; Ponath *et al.*, 1996, *J. Exp. Med.*183:2437-48). All expression constructs were made in pcDNA3 (Invitrogen, CA). Wild-type IL-8RA and IL-8RB cDNA were subcloned into *Hind* III - *Not* I

10 and *EcoR* I - *Not* I sites, respectively. The second initiation site in the IL-8RB sequence (which corresponds to amino acid sequences of MESDS...) was used. Forty-eight hours post-transfection, 0.8 my/ml G418 was added and serial dilutions of cells plated in 96-well plates. After 1-2 weeks, G418-

15 resistant cells were stained by appropriate anti-IL-8R antibodies. High level of expression was enriched either by limiting dilution and re-screening or FACS sorting.

Ligand binding assays were carried out as follows. ^{125}I-labeled human IL8 was purchased from Amersham (Arlington

20 Heights, IL) or DuPont NEN (Boston, MA). For each binding reaction, 60-μl cell cells, washed and resuspended in HBSS containing 5% BSA at 5X10^6/ml, was mixed with 60-μl of 2X reaction mix and incubated at 37°C for 30 min. For most of the experiments, the final concentration of ^{125}I-IL8 in the

25 reaction was 0.1 nM. Non-specific binding was determined in the presence of 100 nM unlabeled IL8. The binding reaction was stopped by transferring 100-μl of the mix to tubes containing 200-μl of Dibutyl phthalate/Bis 2-ethylhexyl phthalate (1:1 mix) and spun at 12 K rpm for 3 min. The

30 supernatant was frozen in dry ice and the cell pellet in the bottom of the tubes was cut off and subjected to counting in a gamma-counter. For Scatchard analysis, 0.05 nM of ^{125}I-IL8 was

added with increasing concentrations of unlabeled IL8. The

data were curve fitted and K_d and B_{max} was calculated using the

computer program Ligand (Munson et al.,1980, *Anal. Biochem.*

107, 220-239).

5   For the Ca^{++} flux assay, neutrophils at 10^7 cells/ml

were incubated with the fluorochrome Fluo-3 (Molecular Probes)

for 30 at RT, washed twice and resuspended at $2x10^6$ cells/ml.

IL8 with or without mAbs was added to Fluo-3 labeled cells and

the internal Ca^{++} concentrations were determined by analyzing

10   FL1 on a FACScan over time.

Of the 14 human anti-rhIL8 monoclonal antibodies isolated

from 2 mice, 10 were specific for rhIL8 and 3 crossreacted

with other chemokines (1 has not yet been tested). Nine of

the 10 IL8-specific mAbs were neutralizing (see Table 28).

15   One of the mAbs, 7C5, that crossreacted with other CXC

chemokines was able to inhibit IL8 binding, but two other

mAbs, 1F8 and 5F10, that only crossreacted with GROa did not

inhibit IL8 binding. mAbs which could inhibit IL8 binding to

IL8RA transfectants could also inhibit IL8 binding to

20   neutrophils and could interfere with IL8-induced Ca^{++} flux.

None of the three mAbs which bound to GROα were able to

inhibit GROα binding.

Based on these specificity data, it appears that

there are at least three different epitopes on IL8 being

25   recognized. The primary one is unique to IL8, a second is

shared between IL8 and GROα, and a third is found more broadly

distributed on CXC chemokines.

Table 28

Specificity and Characterization of Human Anti-IL8 mAbs

mAb	Specific for IL8?*	Cross-Reacts with?	Inhibits rIL8RA Binding?	Inhibits PMN Binding?	Inhibits Ca++ flux?
5 1F8 (14476)	NO	GROα	NO	NO	NO
2D11	YES		YES	YES	NO
2F9	YES		YES	nd	nd
2G1	YES		YES	nd	nd
10 3E5	nd	nd	nd	nd	nd
5E7	YES		YES	nd	nd
5F10	NO	GROα	NO	NO	NO
5H8	YES		YES	nd	nd
2C6	YES		YES	YES	YES
15 (14477)					
2D6	YES		nd	nd	nd
3A1	YES		YES	nd	YES
4D4	YES		YES	nd	nd
7C5	NO	IP10, GROα	YES	nd	nd
20 10A6	YES		YES	nd	nd
2A2**	YES		YES	YES	YES
no mAb	NO		NO	NO	NO

* HuMAbs were tested by ELISA for reactivity with IL8, GROα, GROβ, GROγ, IP10, ENA78 and NAP2.

25 nd = not done

** 2A2 is a murine anti-hIL8 IgG monoclonal antibody that was used as a positive control for these assays. The negative control was no mAb.

     The ability of one the human mAbs, 2C6, to inhibit

30 IL8 was examined in more detail. The mAb was purified from

spent tissue culture fluid over a protein A column and tested

for its ability to inhibit neutrophil chemotaxis and

neutrophil elastase release.

     In the chemotaxis assay, HUVEC or ECV304 cells were

35 adhered to a semi-permeable membrane. Human leukocytes were

added to the chamber on top of the filter and IL8 (with or

without human mAb) was added to the bottom chamber. Cells

which migrated through the endothelial cells adhered to the

filter into the bottom chamber were enumerated with a flow
cytometer using forward and side scatters. For the elastase
release assay, neutrophils were incubated with cytochalasin B
followed by mAb or buffer followed by the elastase substrate.

5   Fluorescence was then measured immediately and 10 min after
the addition of IL8.

Human mAb 2C6 demonstrated dose-dependent inhibition
of both IL8-induced neutrophil chemotaxis and elastase release
(see Figure 98). The IC_{50} values for 2C6 were 270 ng/ml and

10 330 ng/ml, respectively. Both chemotaxis and elastase release
were completely inhibited by as little as 1 μg/ml of mAb.
This human mAb is slightly more potent than the most potent
murine anti-IL8 mAb examined to date.

15   ***

The foregoing description of the preferred
embodiments of the present invention has been presented for
purposes of illustration and description. They are not

20 intended to be exhaustive or to limit the invention to the
precise form disclosed, and many modifications and variations
are possible in light of the above teaching. It will be
apparent that certain changes and modifications may be
practiced within the scope of the claims.

25 All publications and patent applications herein are
incorporated by reference to the same extent as if each
individual publication or patent application was specifically
and individually indicated to be incorporated by reference.
Commonly assigned applications U.S.S. N. 08/728,463 filed

30 October 10, 1996, U.S.S.N. 08/544,404 filed 10 October 1995,
U.S.S.N. 08/352,322, filed 7 December 1994, U.S.S.N.
08/209,741 filed 9 March 1994, U.S.S.N. 08/165,699 filed 10

December 1993 and U.S.S.N. 08/161,739 filed 03 December 1993,
which is a continuation-in-part of 08/155,301 filed 18
November 1993, WO92/03918, USSN 07/810,279 filed 17 December
1991, USSN 07/853,408 filed 18 March 1992, USSN 07/904,068
5   filed 23 June 1992, USSN 07/990,860 filed 16 December 1992,
WO93/12227, and USSN 08/053,131 filed 26 April 1993 are each
incorporated herein by reference.

WHAT IS CLAIMED IS:

1.   A hybridoma comprising a B cell obtained from a
transgenic mouse having a genome comprising a human heavy
5   chain transgene and a human light chain transgene, said B cell
fused to an immortalized cell suitable to generate a
hybridoma, wherein said hybridoma produces a detectable amount
of an immunoglobulin that specifically binds interleukin-8.

10   2.   The hybridoma of Claim 1 wherein the
immunoglobulin specifically binds GROα.

3.   The hybridoma of Claim 1, wherein the
immunoglobulin has an affinity constant (K_a) of at least 2 x
15   10^9 M^{-1} for binding human interleukin-8.

4.   The hybridoma of Claim 1, wherein the affinity
constant is at least 2 x 10^{10} M^{-1}.

20   5.   The hybridoma of Claim 3 wherein the
immunoglobulin is selected from the group consisting of 1F8,
2D11, 2F9, 2G1, 3E5, 5E7, 5F10, 5H8, 2C6, 2D6, 3A1, 4D4, 7C5,
and 10A6.

25   6.   A composition comprising a substantially pure
immunoglobulin produced by the hybridoma of claim 3.

7.   A composition comprising a substantially pure
human monoclonal antibody, wherein said antibody has an
30   affinity constant (K_a) of at least 2 x 10^9 M^{-1} for binding human
interleukin-8, and wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light

5 chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a C_L gene segment; and

a human sequence heavy chain composed of (1) a heavy chain variable region having a polypeptide sequence which is

10 substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

8. The composition of claim 7 wherein the human monoclonal antibody specifically binds GROα.

9. A method of preventing efflux of neutrophils from vasculature in a patient, comprising administering an effective amount of a human monoclonal antibody having an affinity constant (K_a) of at least 2×10^9 M^{-1} for binding human interleukin-8.

10. A method of treating reperfusion injury comprising administering comprising administering to a patient a therapeutically effective dose of a human monoclonal antibody having an affinity constant (K_a) of at least 2×10^9 M^{-1} for binding human interleukin-8.

11. A method of suppressing a T-helper cell dependent immune response in a primate, comprising

administering a therapeutically effective dose of a human monoclonal antibody having an affinity constant (K_a) of at least 2×10^9 M^{-1} for binding human CD-4.

12. The method of claim 11 wherein the primate is a chimpanzee.

13. The method of claim 11 wherein the antibody comprises a VH4-34 segment, a JH5 segment, a heavy chain CDR3 region comprising the sequence VINWFDP, a VkL19 segment, a Jk2 segment, and a light chain CD3 region comprising the sequence QQANSFPYT.

14. The method of claim 13 wherein the antibody is 6G5.

15. The method of claim 11 wherein the antibody comprises a VH5-51 segment, a JH2 segment, a heavy chain CDR3 region comprising the sequence PANWNWYFVL, a VkL18 segment, a Jk4 segment, and a light chain CD3 region comprising the sequence QQFISYPQLT.

16. The method of claim 15 wherein the antibody is 1G2.
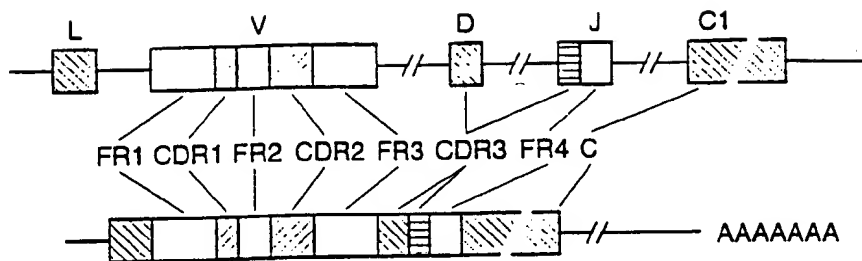
FIG. 1
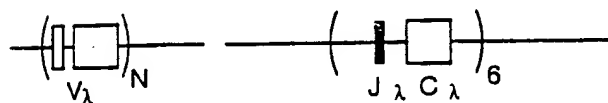
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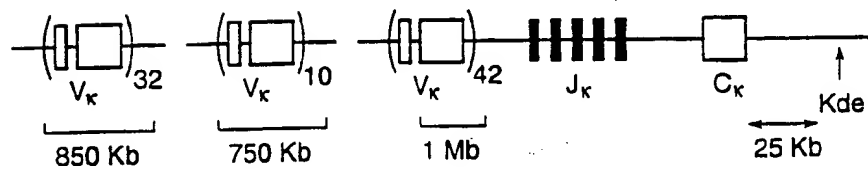
FIG. 3

FIG. 4
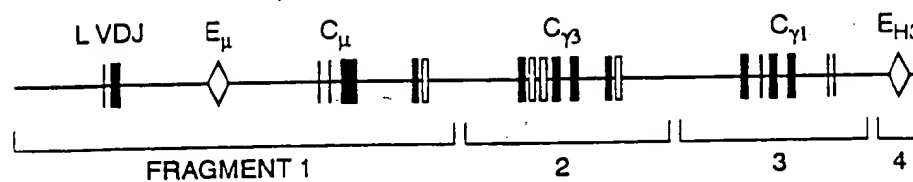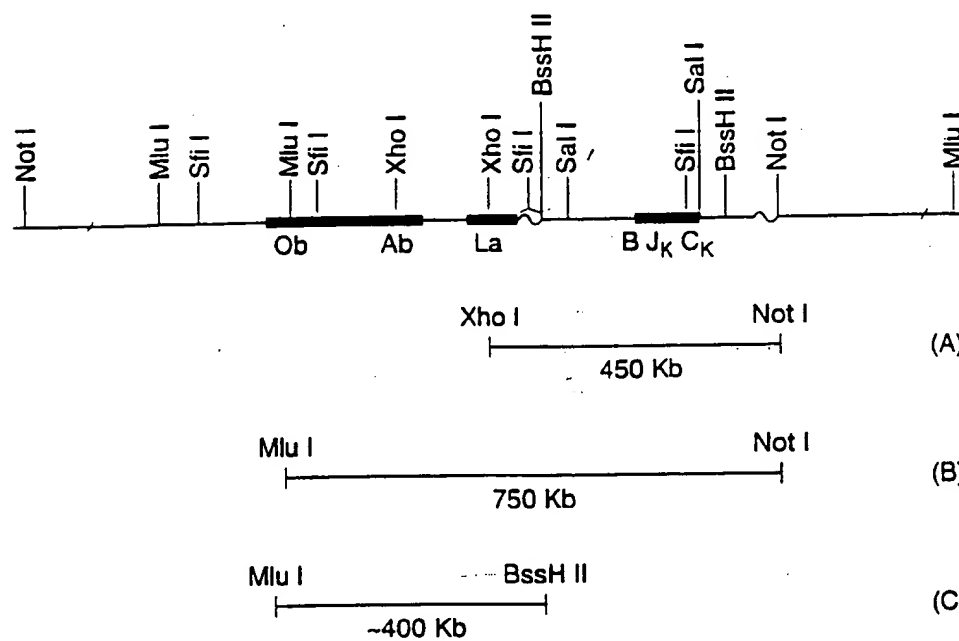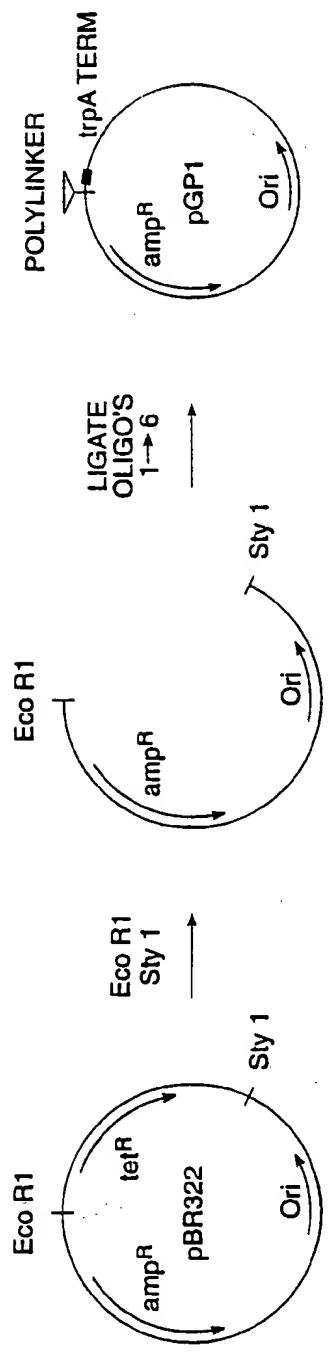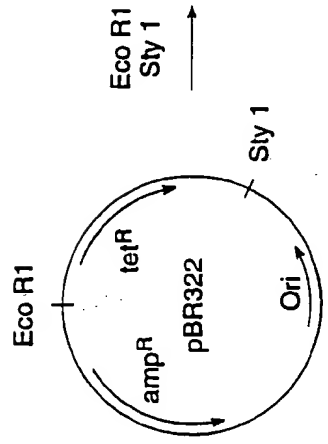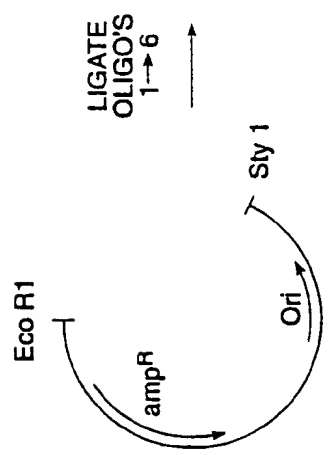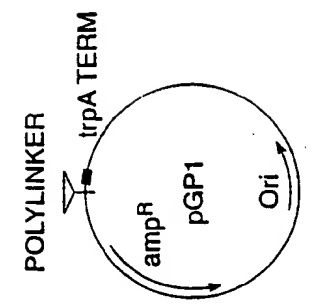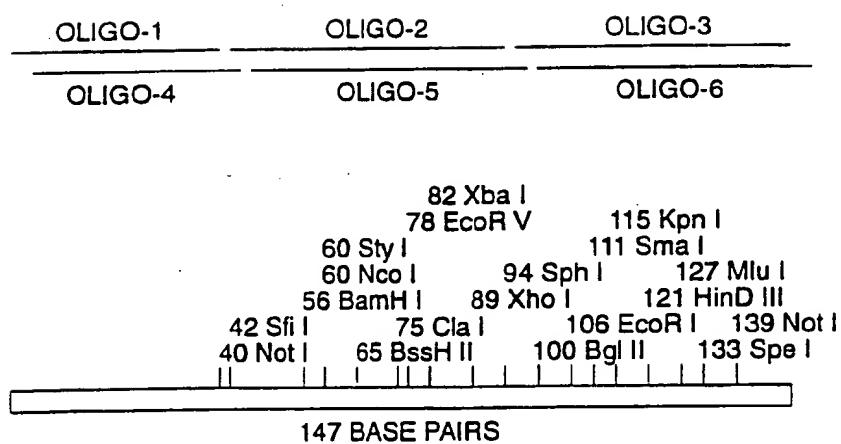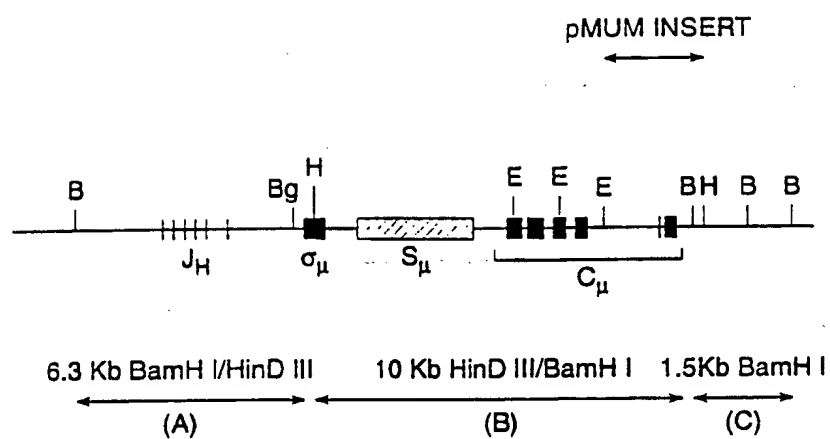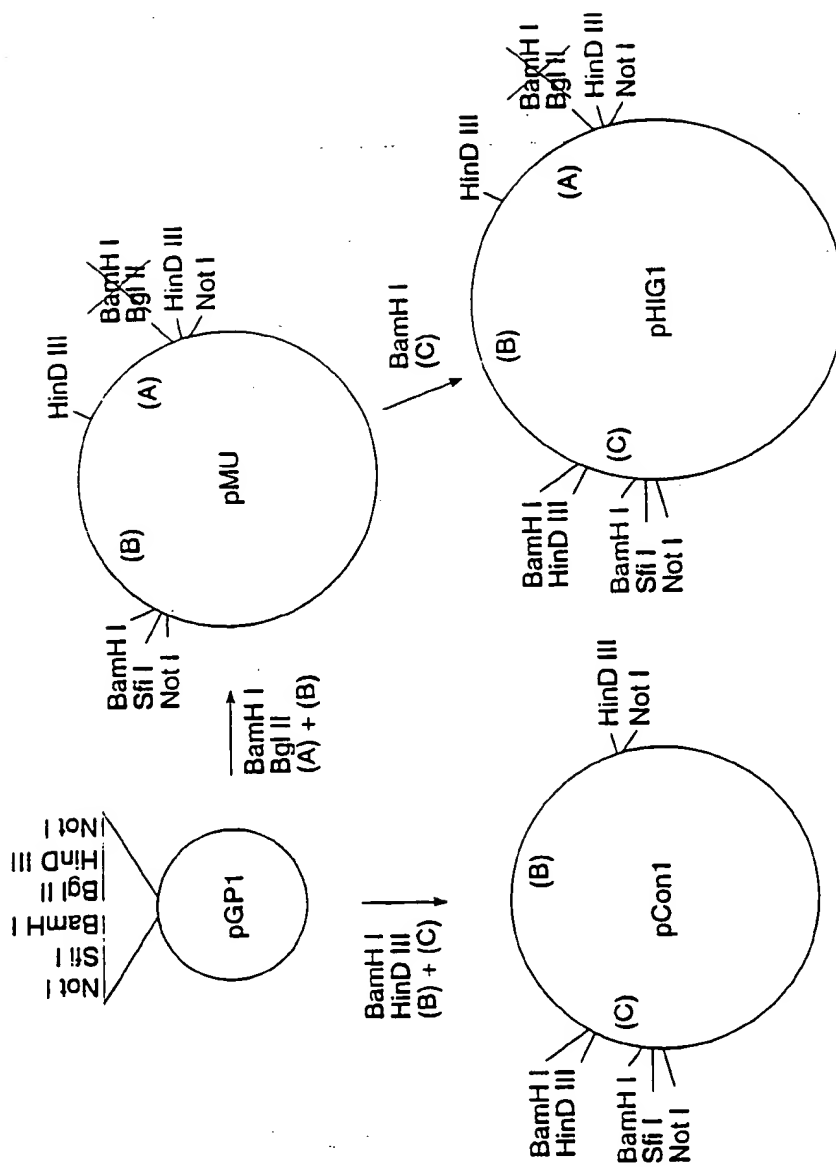
FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

FIG. 10

HUMAN C$_{\gamma 1}$ GENE

FIG. 11

pHIG1/Sfi I
+
pREG2 Sfi I
INSERT

Sfi I

C$_\mu$

C$_{\gamma l}$ pHI G3'

J$_H$

3' ENH

Sfi I   Spe I
Not I   Not I

31.5 Kb Not I/Spe I INSERT

pCON1/Sfi I
+
pREG2 Sfi I
INSERT

Sfi I

C$_\mu$

C$_{\gamma l}$ pCON

3' ENH

Sfi I   Spe I
Not I   Not I

25 Kb Not I/Spe I INSERT

FIG. 12

FIG. 13

FIG. 14

FIG. 15

FIG. 16

FIG. 17
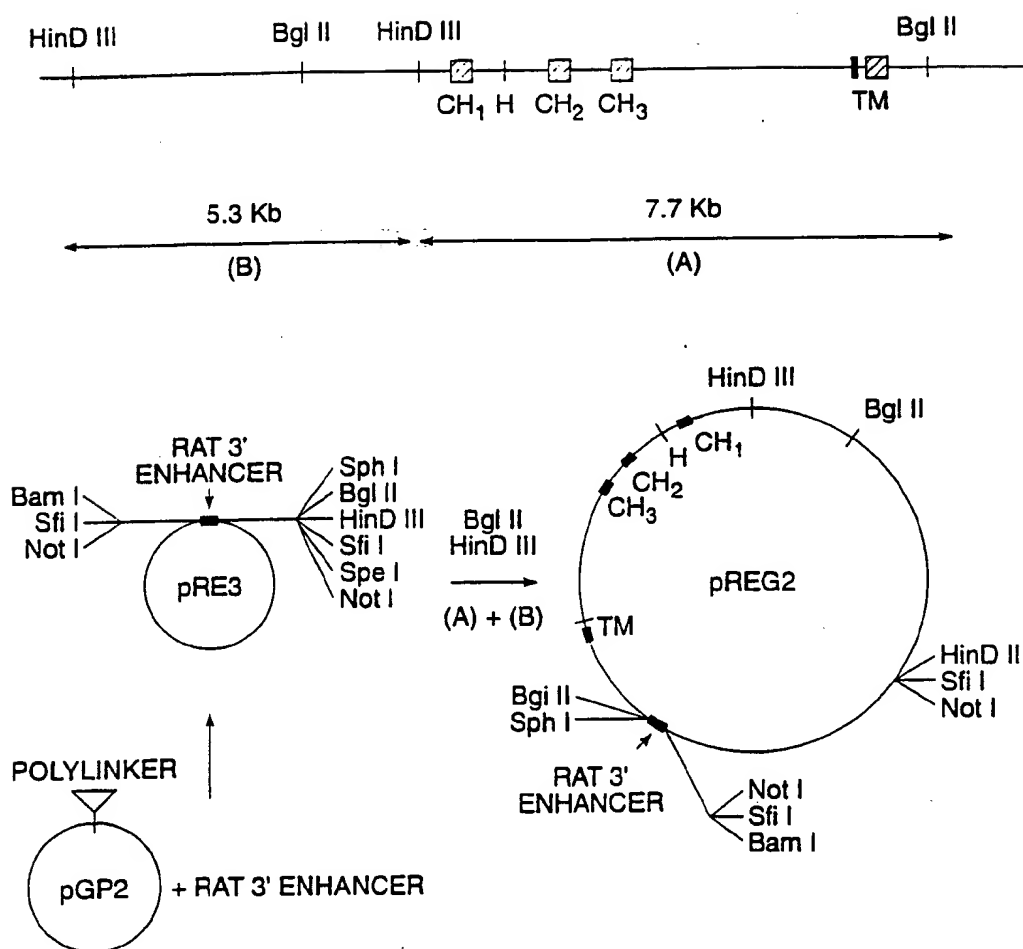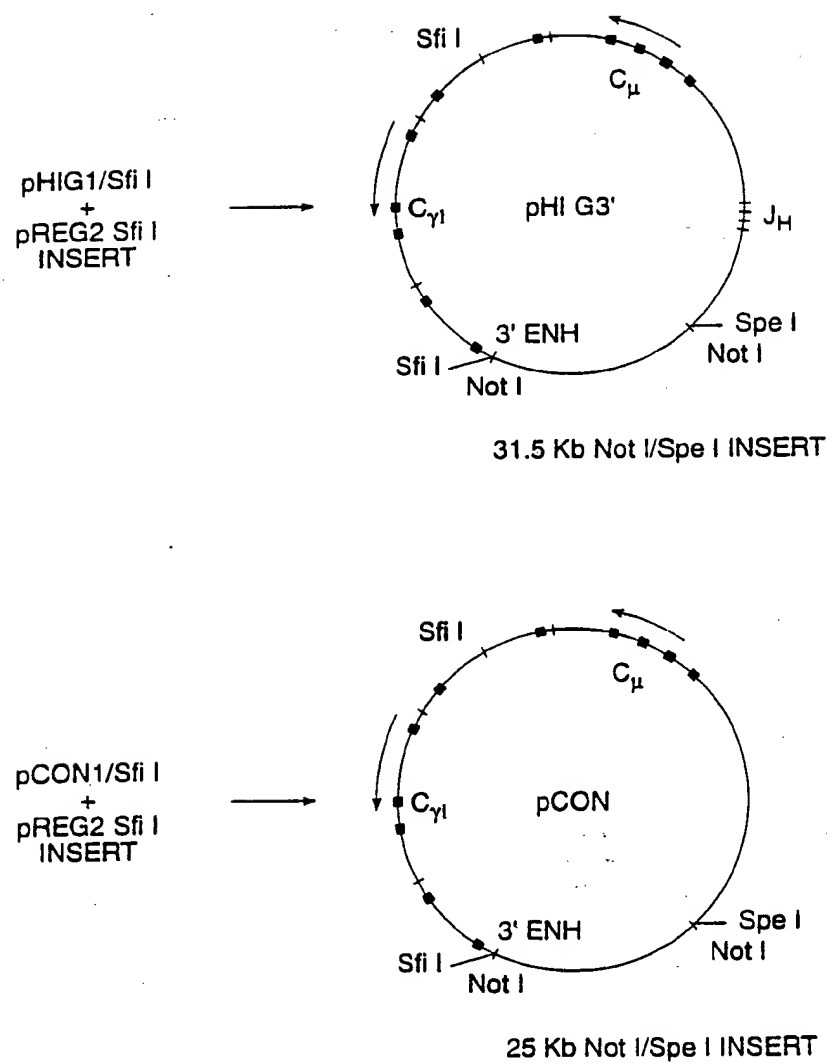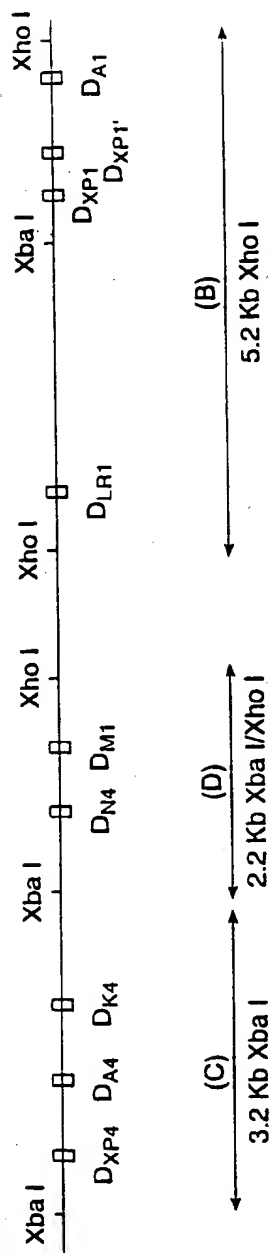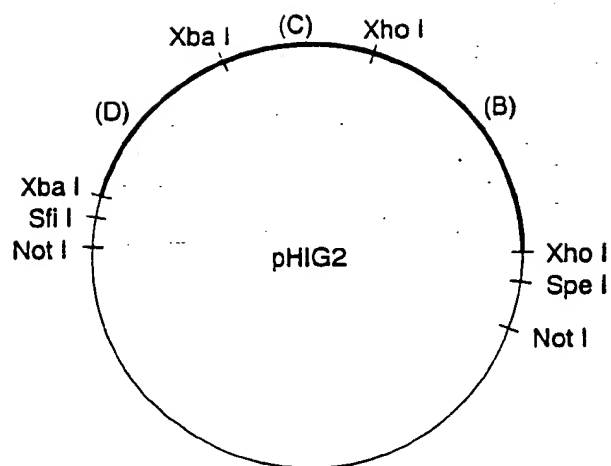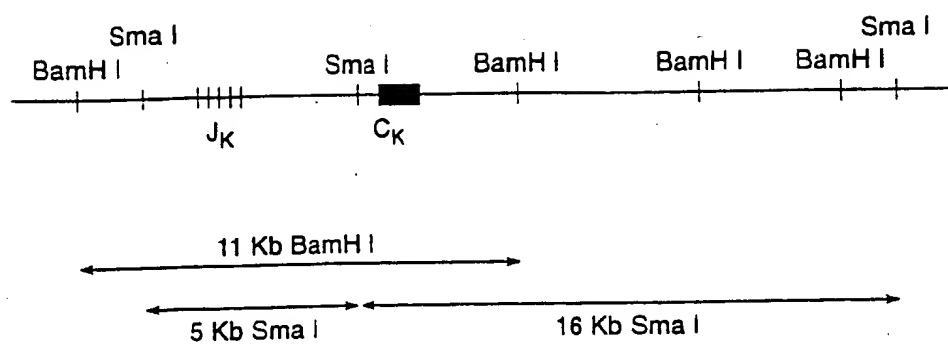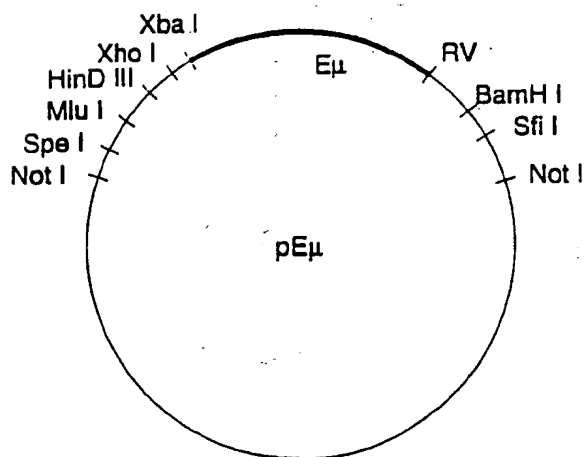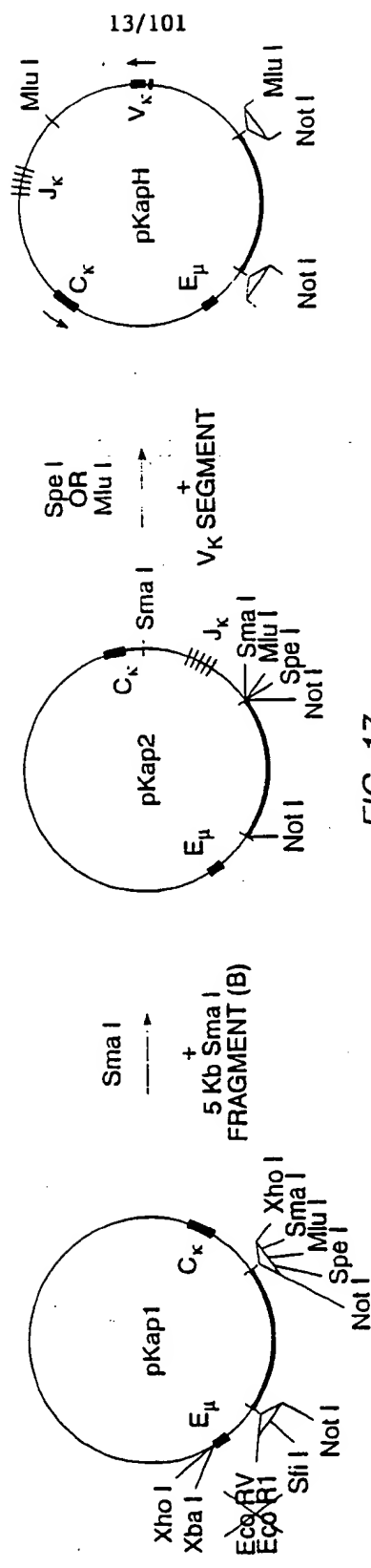
MOUSE HEAVY CHAIN LOCUS
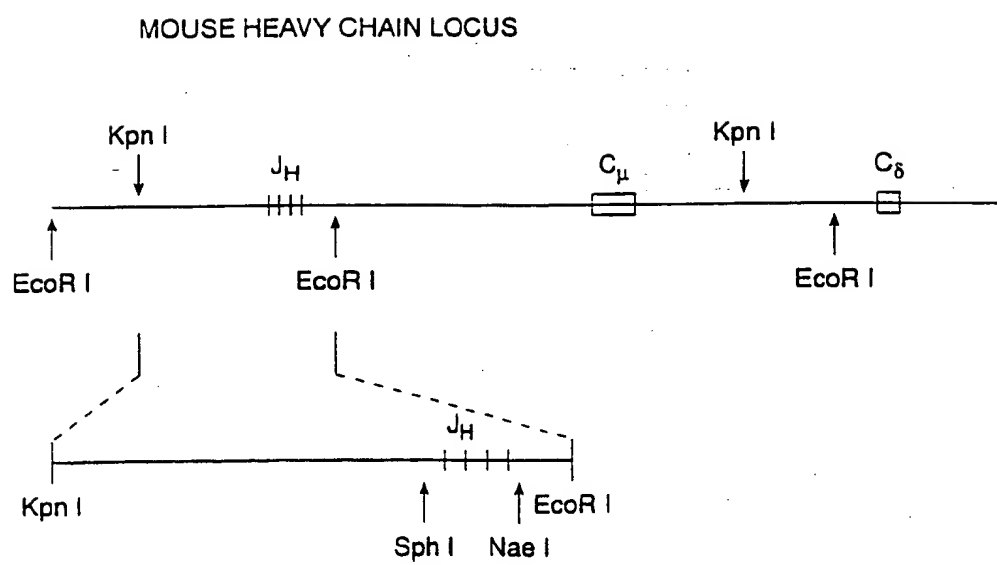
FIG. 18A

FIG. 18B

FIG. 18C

FIG. 18D

MOUSE KAPPA GENE

FIG. 19A
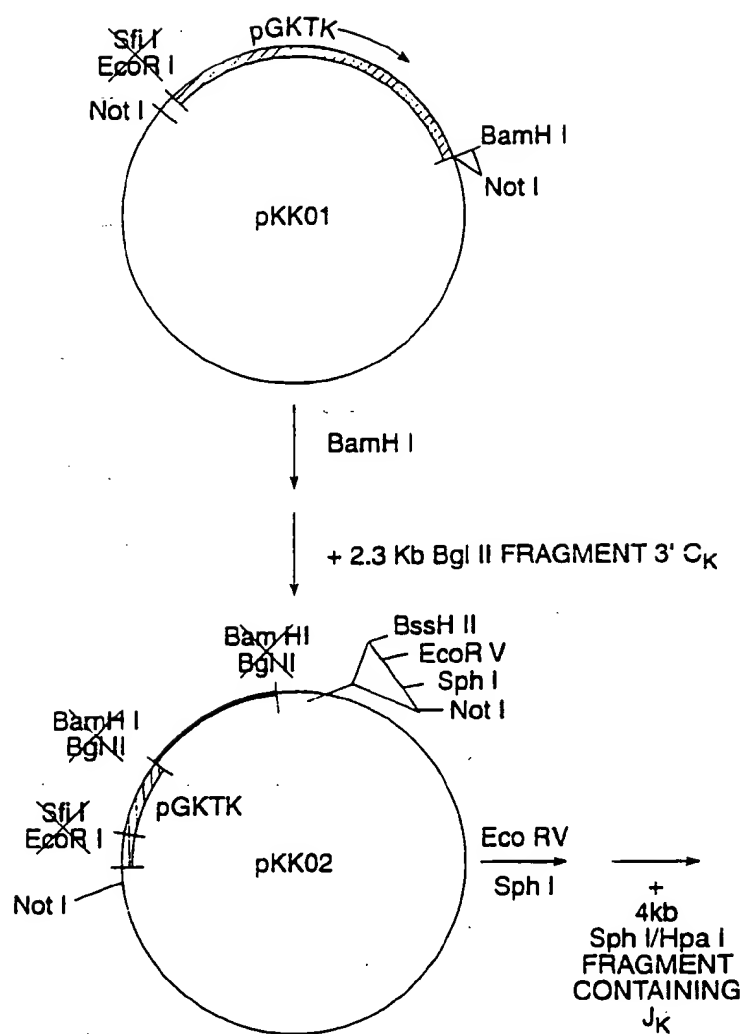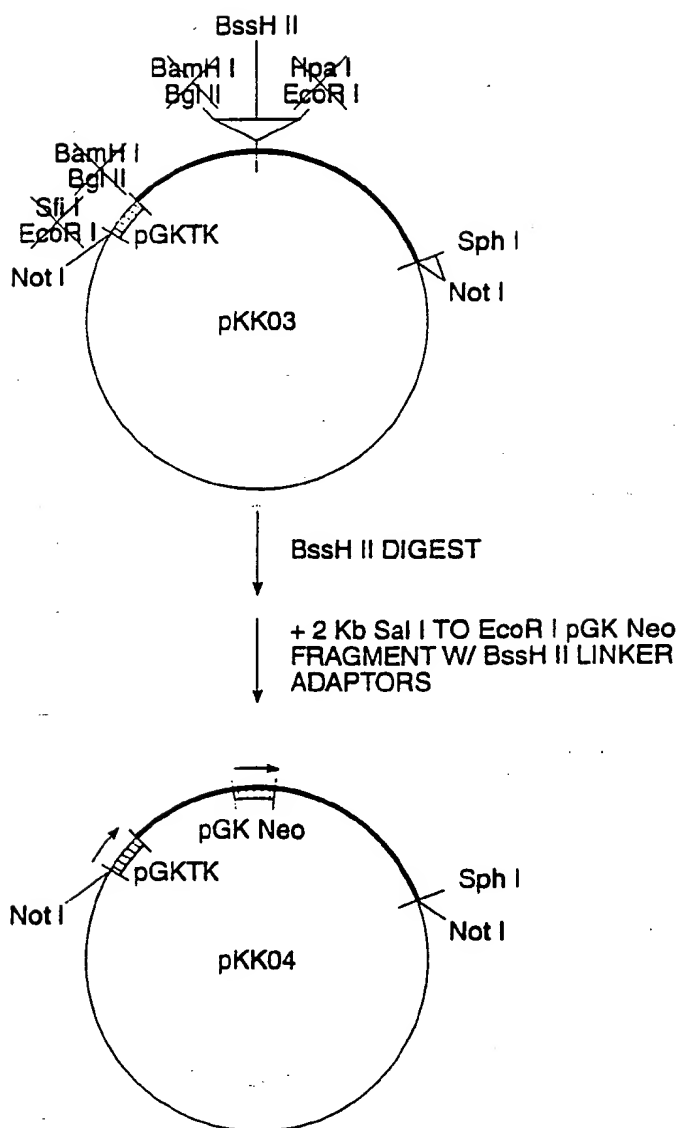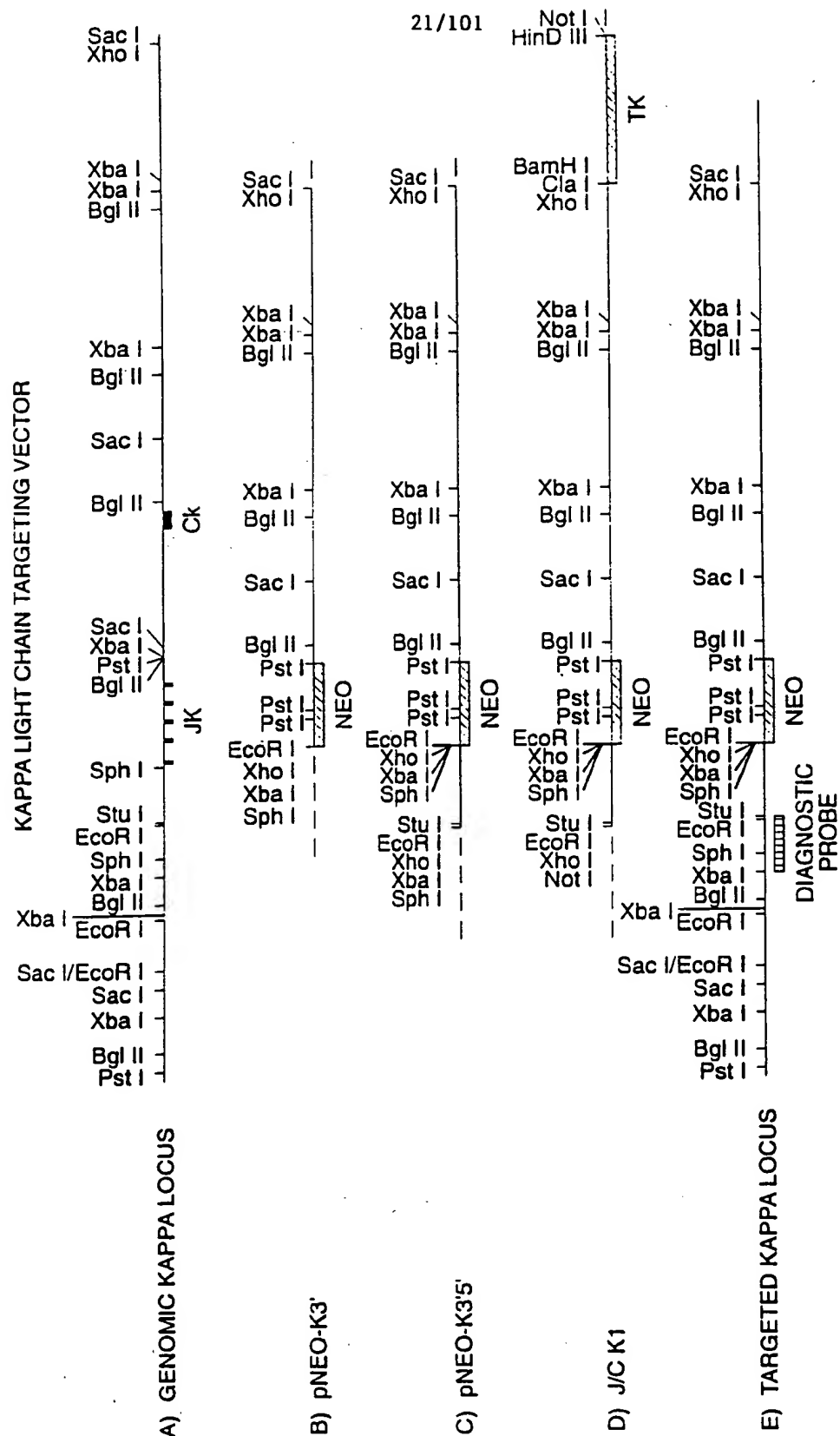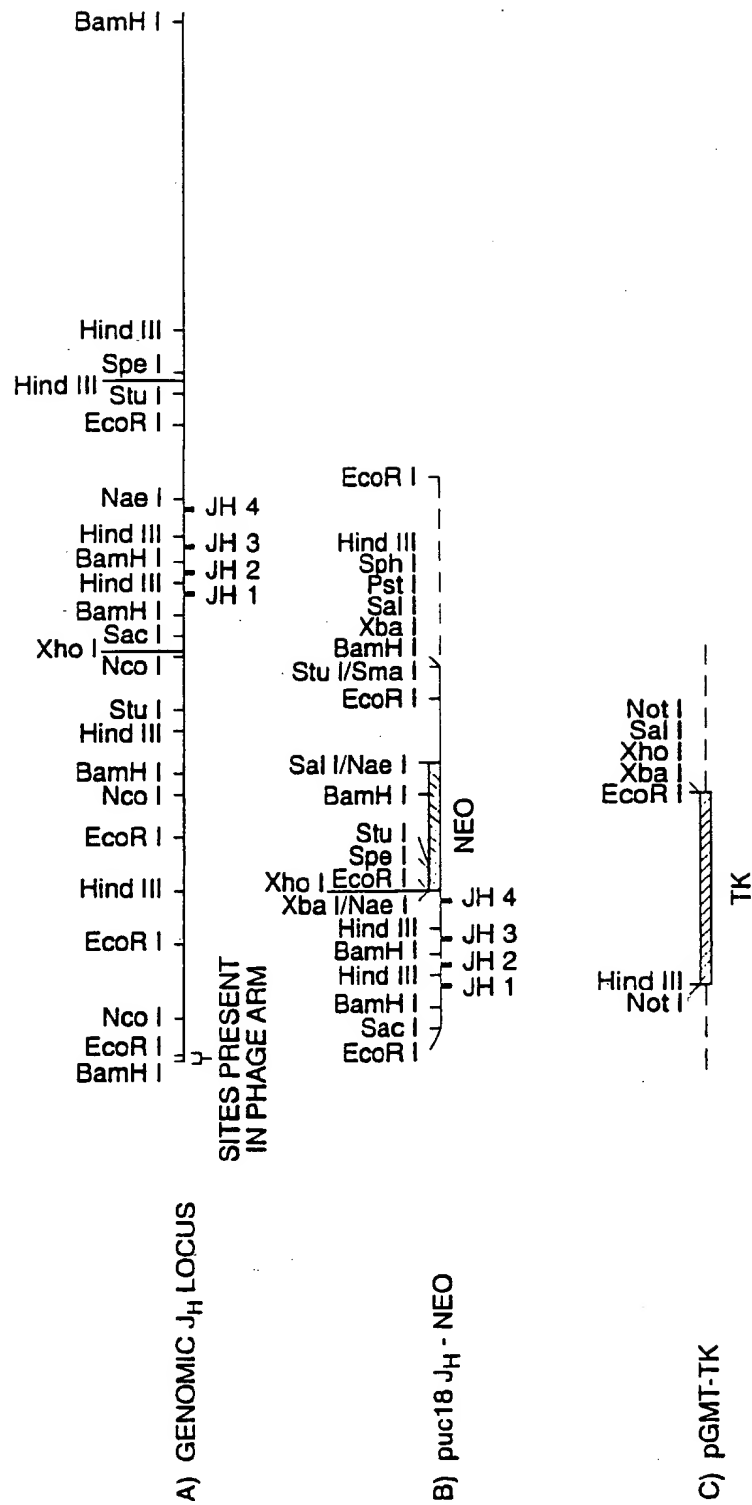
FIG. 19B

FIG. 19C

FIG. 20

KAPPA LIGHT CHAIN TARGETING VECTOR

A) GENOMIC KAPPA LOCUS

B) pNEO-K3'

C) pNEO-K3'5'

D) J/C K1

E) TARGETED KAPPA LOCUS

FIG. 21A-C

MOUSE HEAVY CHAIN TARGETING VECTOR

A) GENOMIC J_H LOCUS

B) puc18 J_H - NEO

C) pGMT-TK

MOUSE HEAVY CHAIN TARGETING VECTOR

D) pGMT-TK-J_H5'

Not I
Sal I
Xho I

Stu I
Hind III

BamH I

EcoR I

Hind III

EcoR I

Xba I
EcoR I

TK

Hind III
Not I

E) J_HKO1

Not I
Sal I
EcoR I/Xho I

Sal I/Nae I
BamH I

NEO

EcoR I/Xho I

Stu I
Hind III

BamH I

EcoR I

Hind III

EcoR I

Xba I
EcoR I

TK

Hind III
Not I

F) TARGETED J_H LOCUS

BamH I

Hind III
Spe I
Hind III   Stu I
EcoR I

DIAGNOSTIC PROBE

Sal I/Nae I
BamH I

NEO

EcoR I/Xho I
Nco I

Stu I
Hind III

BamH I
Nco I

EcoR I

Hind III

EcoR I

Nco I
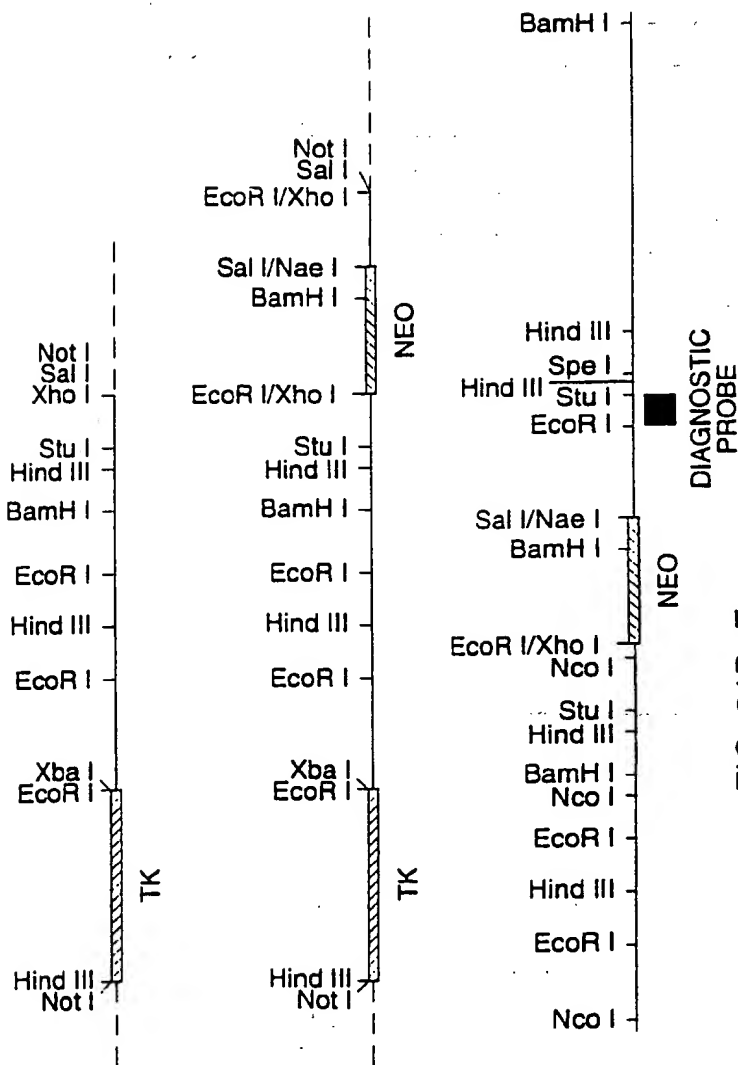
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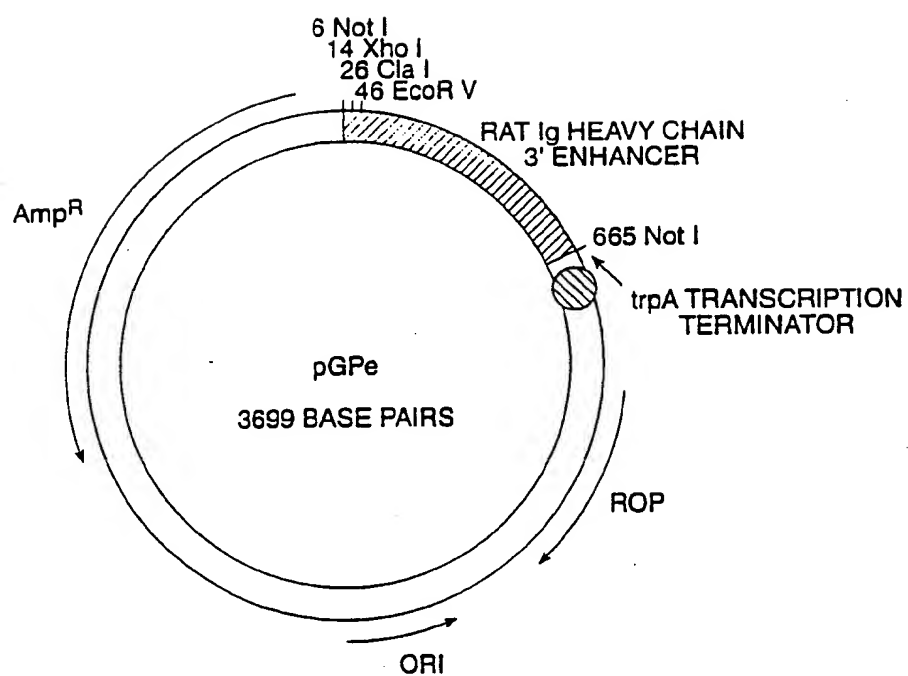
FIG. 22

International application No.

PCT/US97/21803

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/9.2, 178.1, 93.1, 130.1; 435/69.7, 69.1, 375; 530/387.1; 514/2
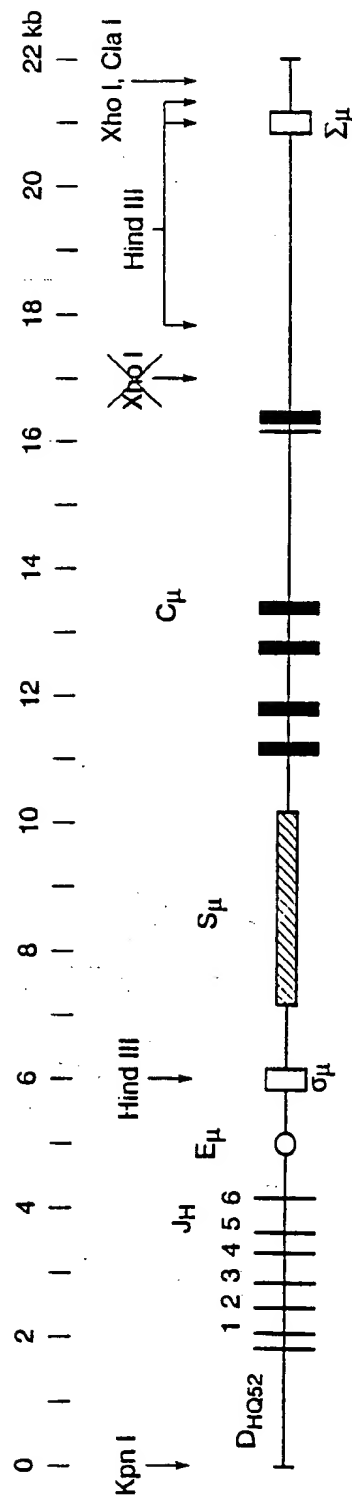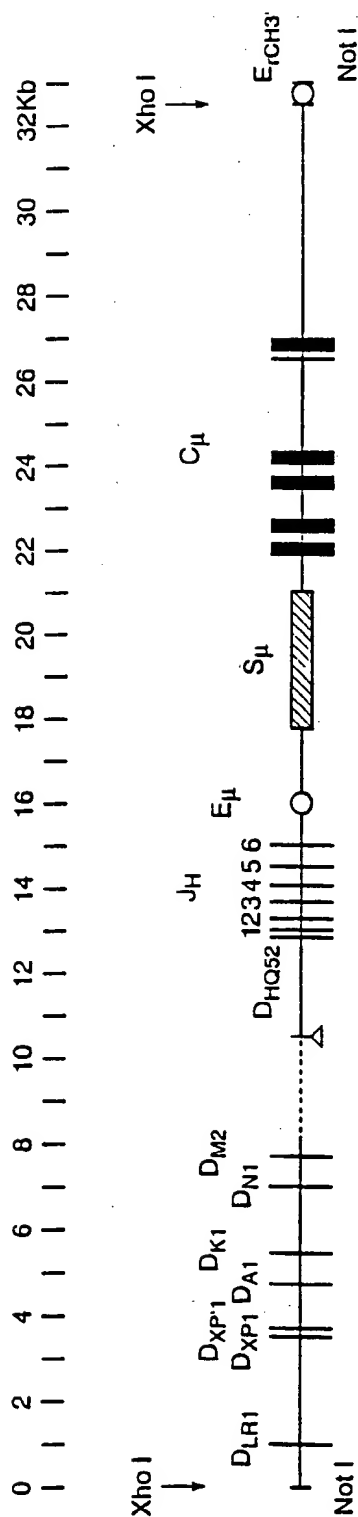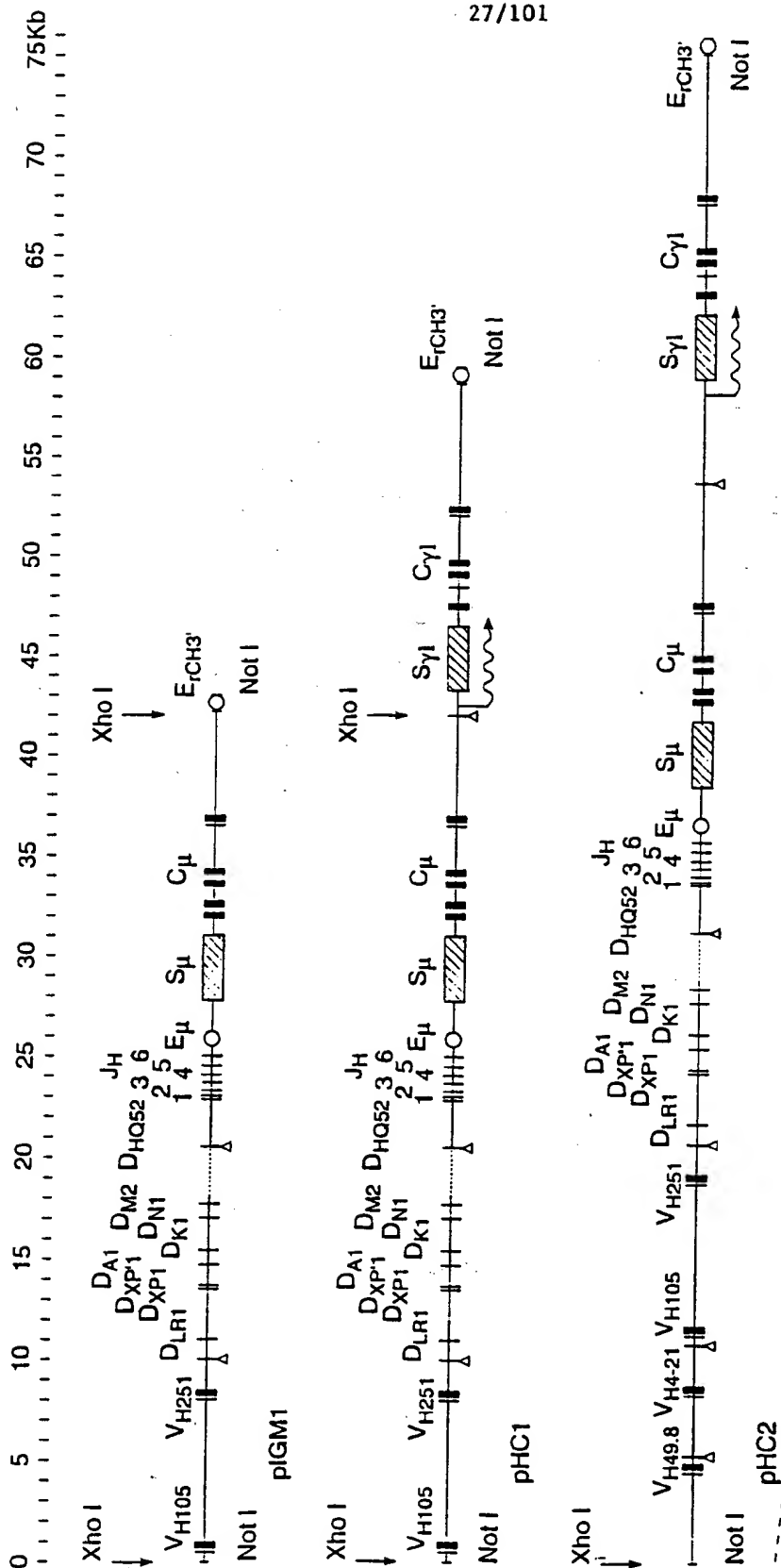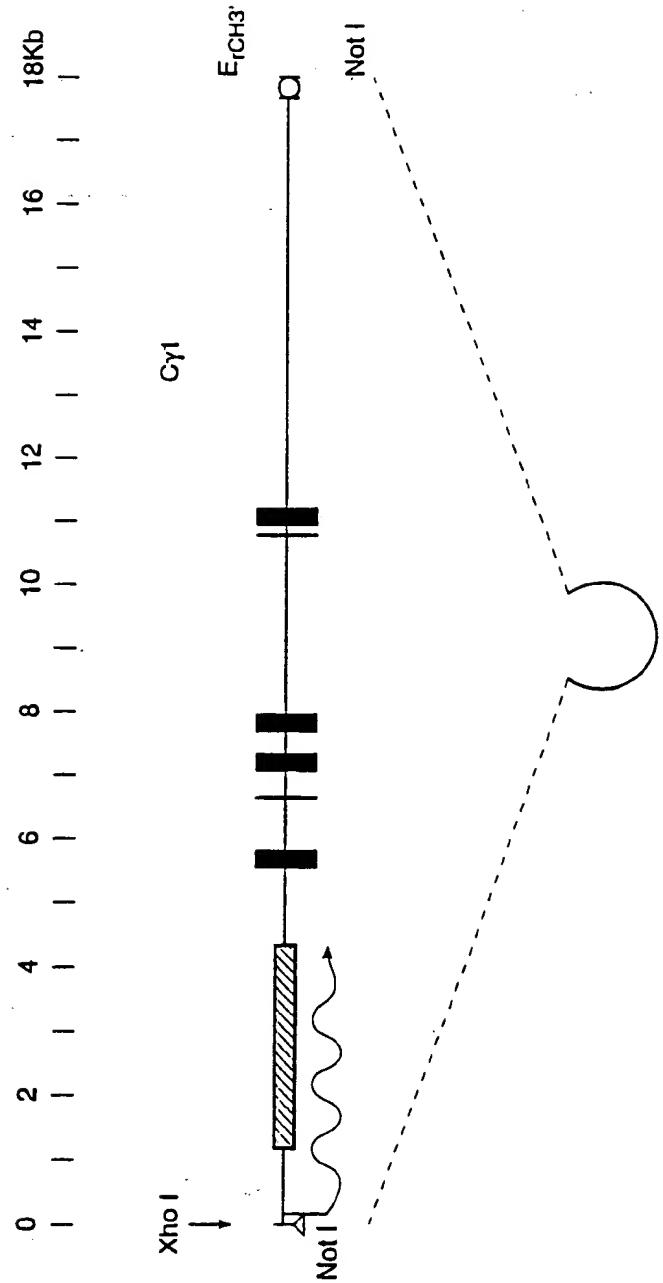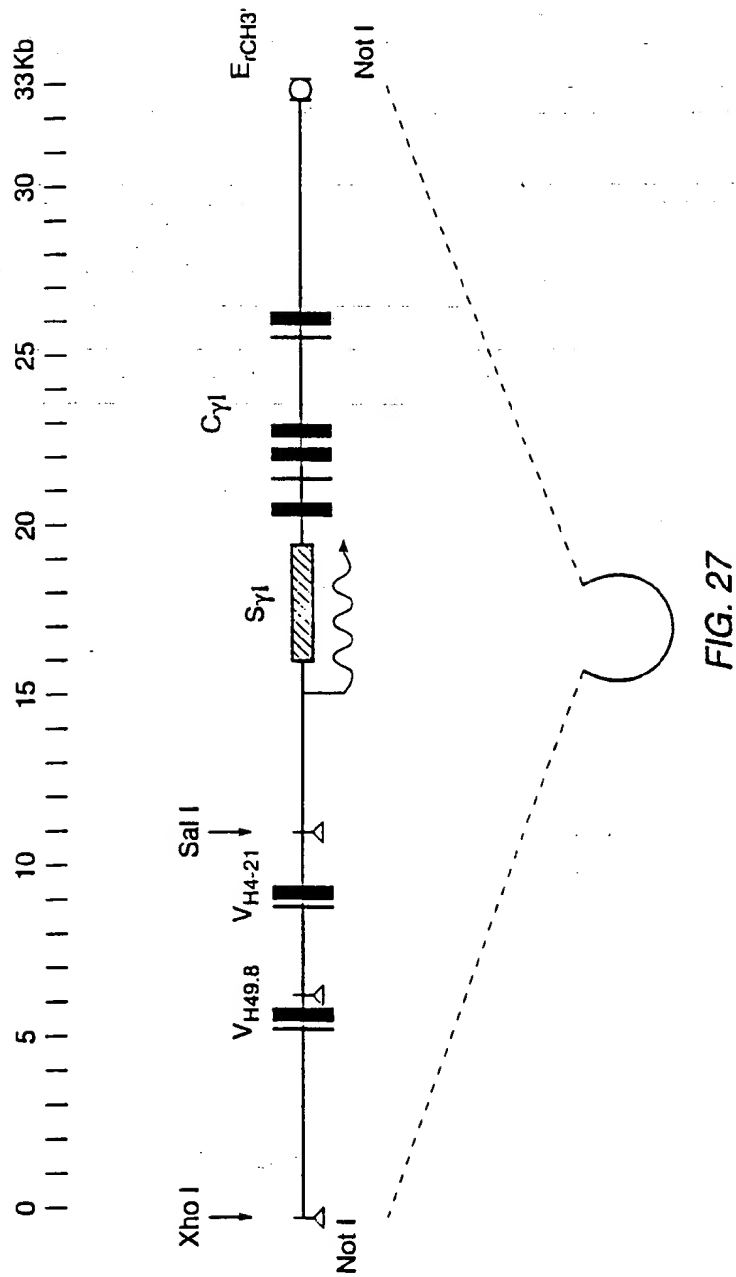
FIG. 23

FIG. 24

FIG. 25

FIG. 26

FIG. 27
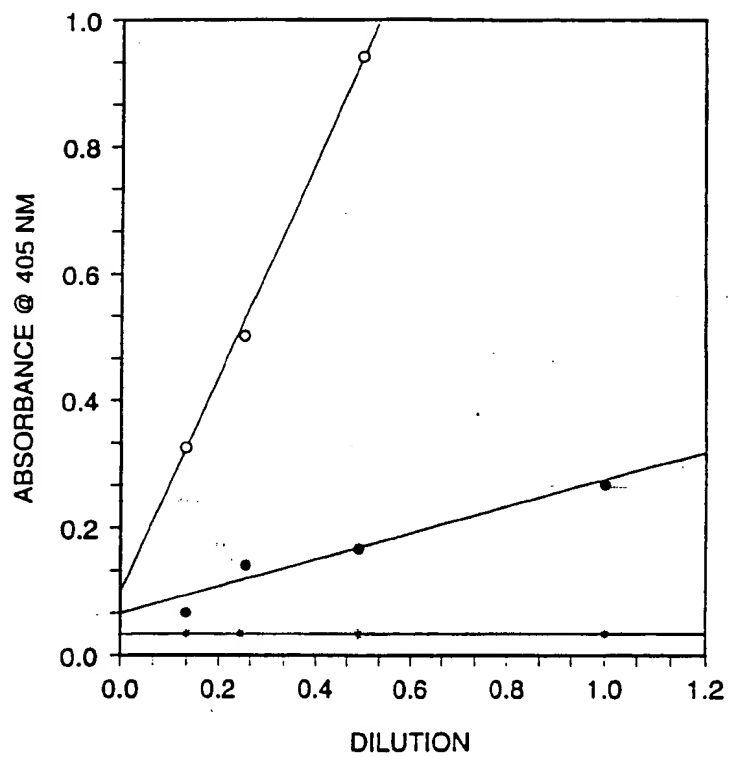
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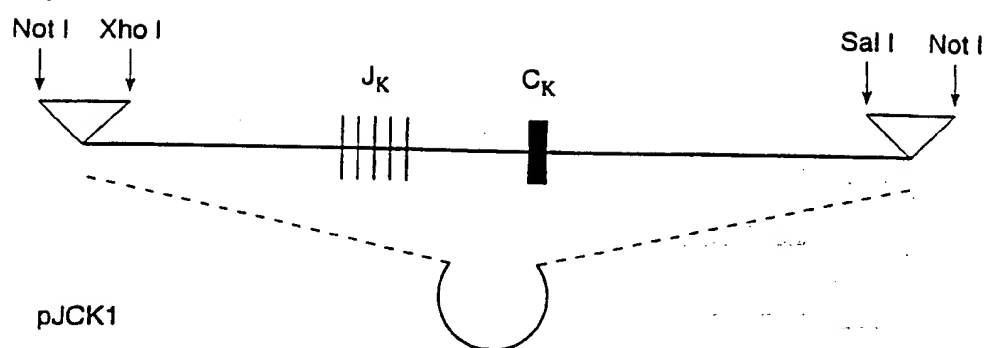
FIG. 29
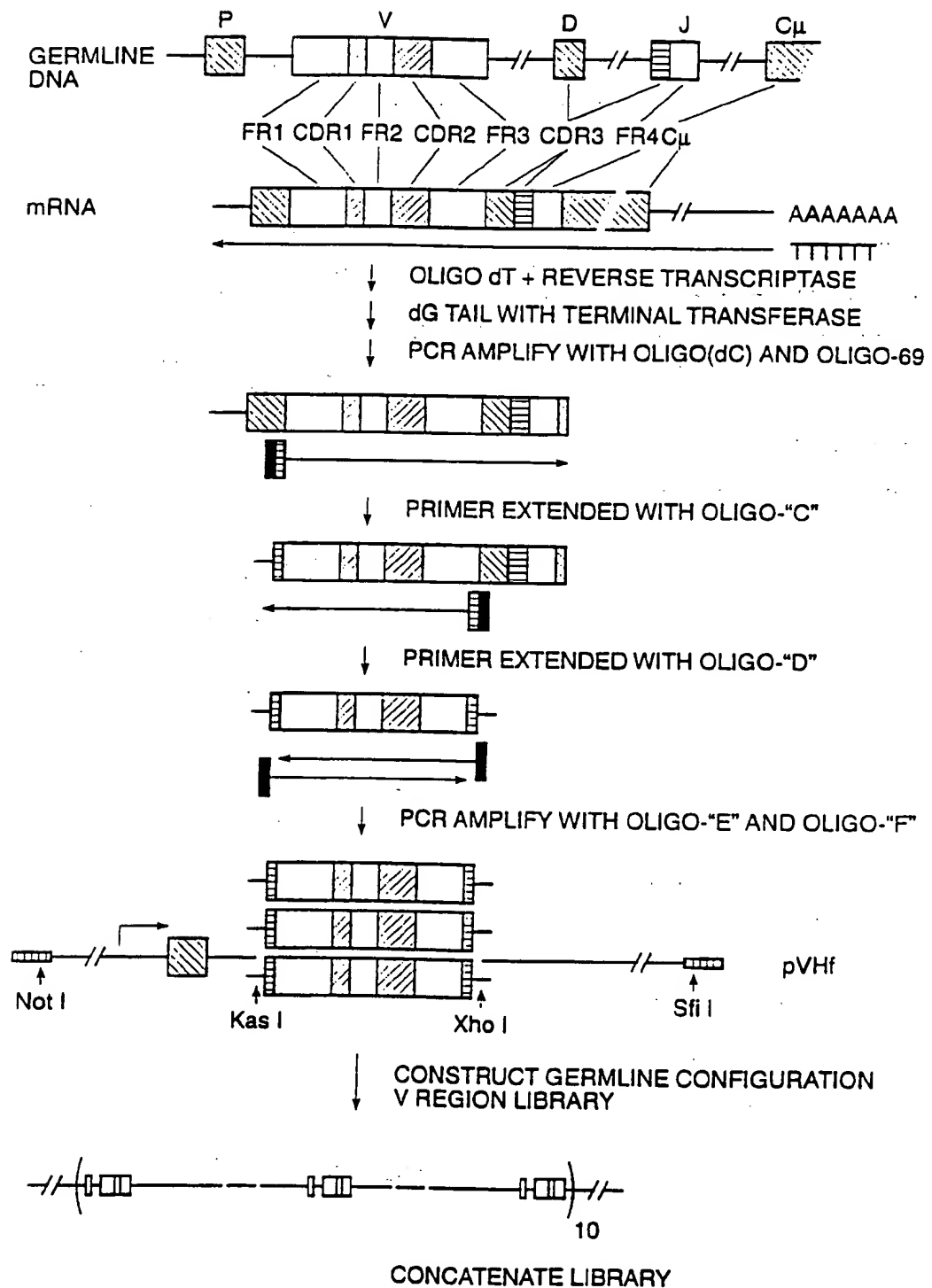
SYNTHETIC HEAVY CHAIN VARIABLE REGION

FIG. 30

FIG. 31

FIG. 32

FIG. 33

FIG. 34

FIG. 35

FIG. 36

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Medline abstract number 96/47983, JI et al. Flow cytometry analysis of the neutralization effect of anti-IL8 monoclonal antibodies on IL-8 activated human granulocytes, Journal of Experimental Biology, Volume 28, Number 3, September 1995, see entire abstract.	1-16
Y	US 5,545,806 A (LONBERG et al.) 13 August 1996, entire patent.	1-16

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 5/28, 5/24, 5/10; C07K 16/00; A61K 39/00
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.2, 178.1, 93.1, 130.1; 435/69.7, 69.1, 375; 530/387.1; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, CAPLUS, APS
search terms: antibody, interleukin 8, hybridoma, treatment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEKIDO et al. Prevention of Lung Reperfusion Injury in Rabbits by a Monoclonal Antibody Against Interleukin-8. Nature. 14 October 1993, Vol. 365, pages 654-657, see entire document.	1-16
Y	KURDOWSKA et al. An anti-interleukin 8 monoclonal antibody that interferes with the binding of interleukin 8 to cellular receptors and the activation of human blood neutrophils. Hybridoma. 1995, Vol. 14, No. 3, pages 225-233, see entire document.	1-16
Y	WO 96/02576 A1 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 01 February 1996, entire patent.	1-16

| X | Further documents are listed in the continuation of Box C. | ☐ | See patent family annex. |

*	Special categories of cited documents:
'A'	document defining the general state of the art which is not considered to be of particular relevance
'B'	earlier document published on or after the international filing date
'L'	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O'	document referring to an oral disclosure, use, exhibition or other means
'P'	document published prior to the international filing date but later than the priority date claimed

'T'	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'X'	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'Y'	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
'A'	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 FEBRUARY 1998	1 7 MAR 1998

Name and mailing address of the ISA/US	Authorized Officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KAREN M. HAUDA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Fig. 98

Fig. 97

Human IgM anti-IL8 Responses

TT 9/6/96 anti-CD4 Preps

TT 10/2/96 anti-CD4 Preps
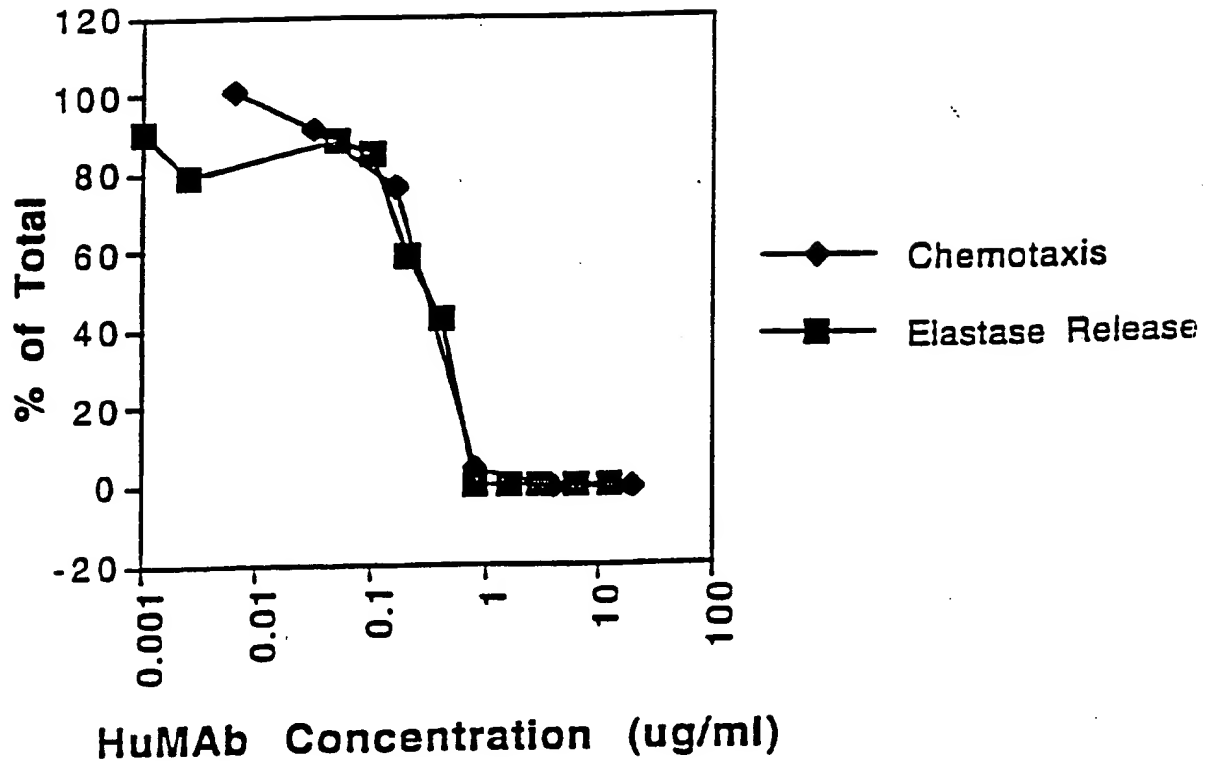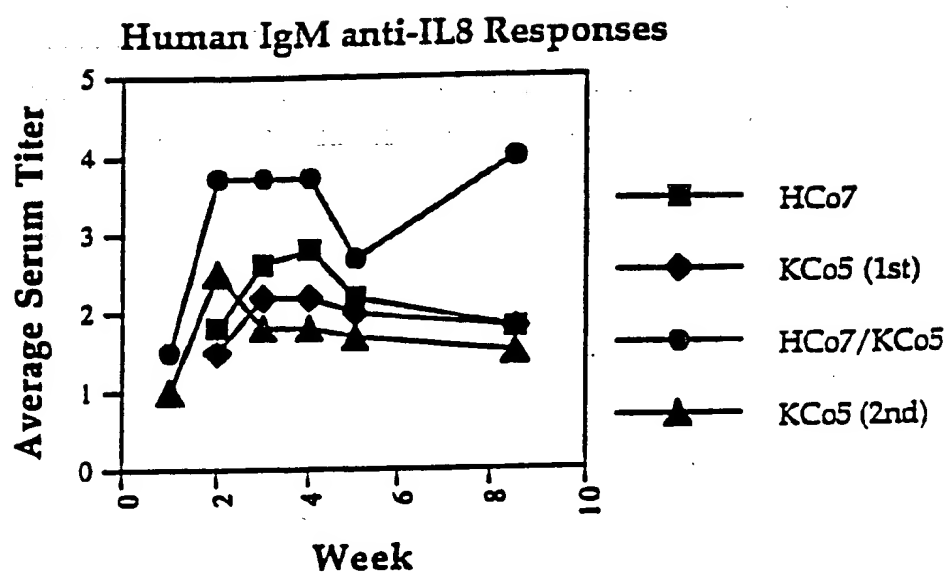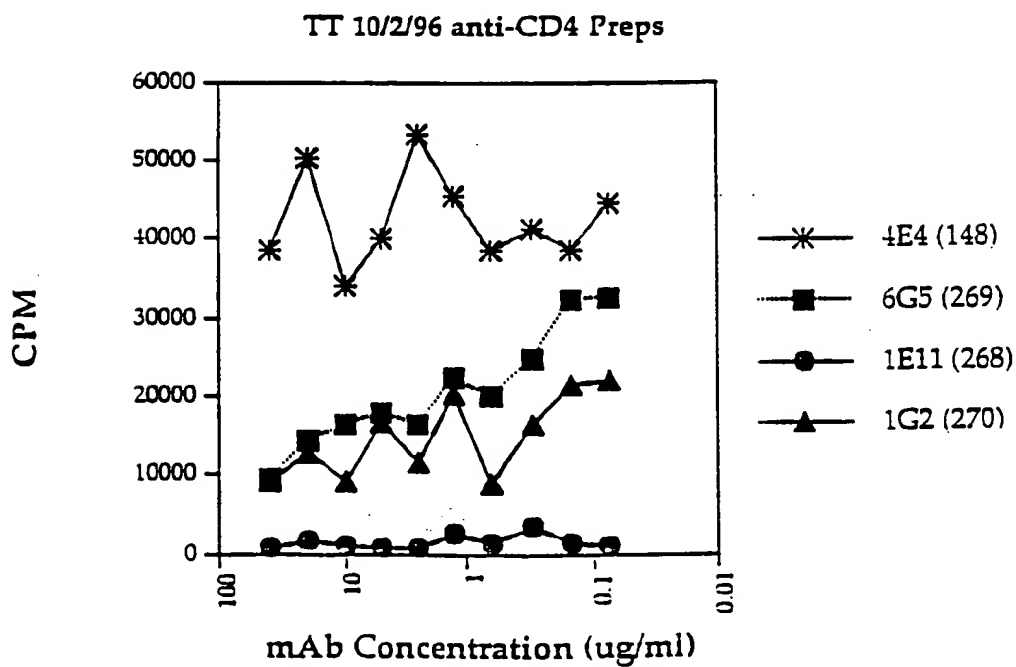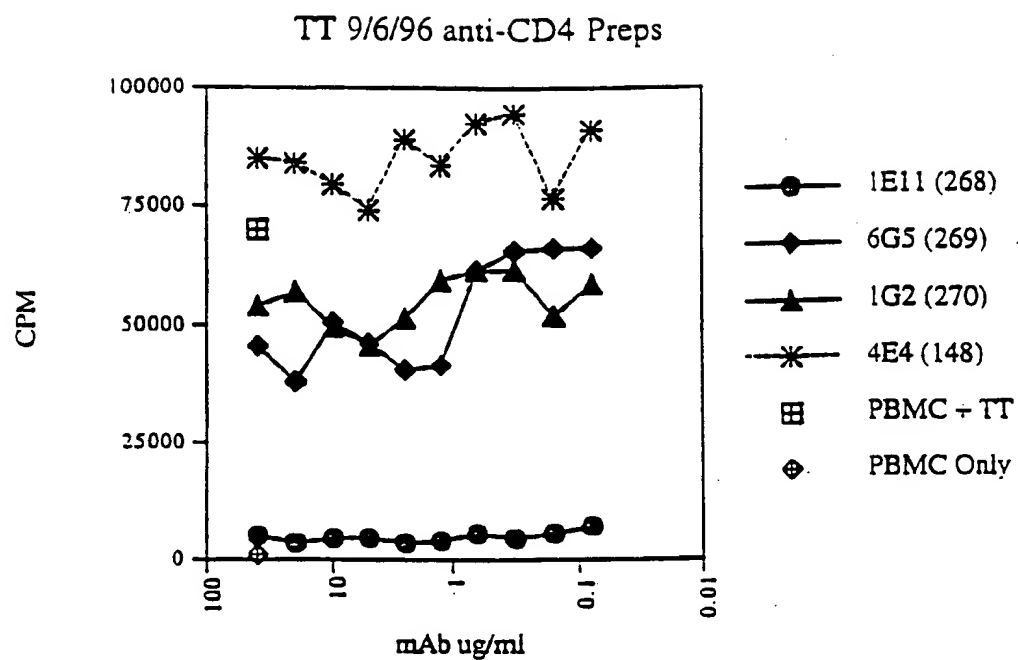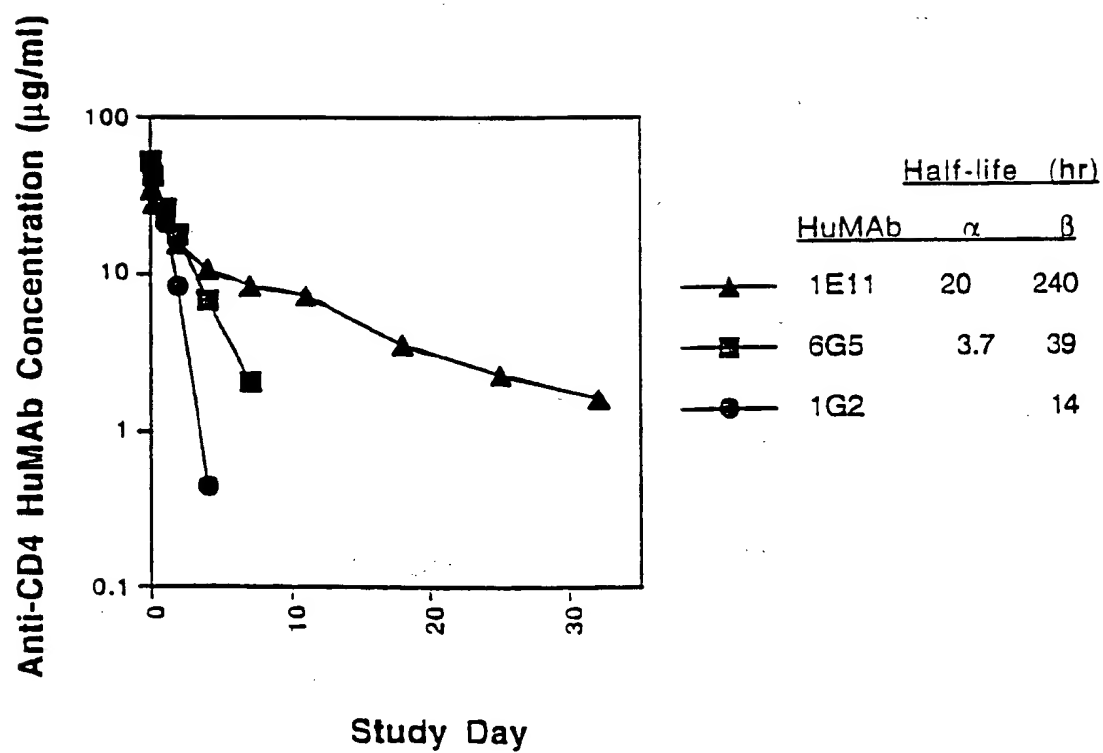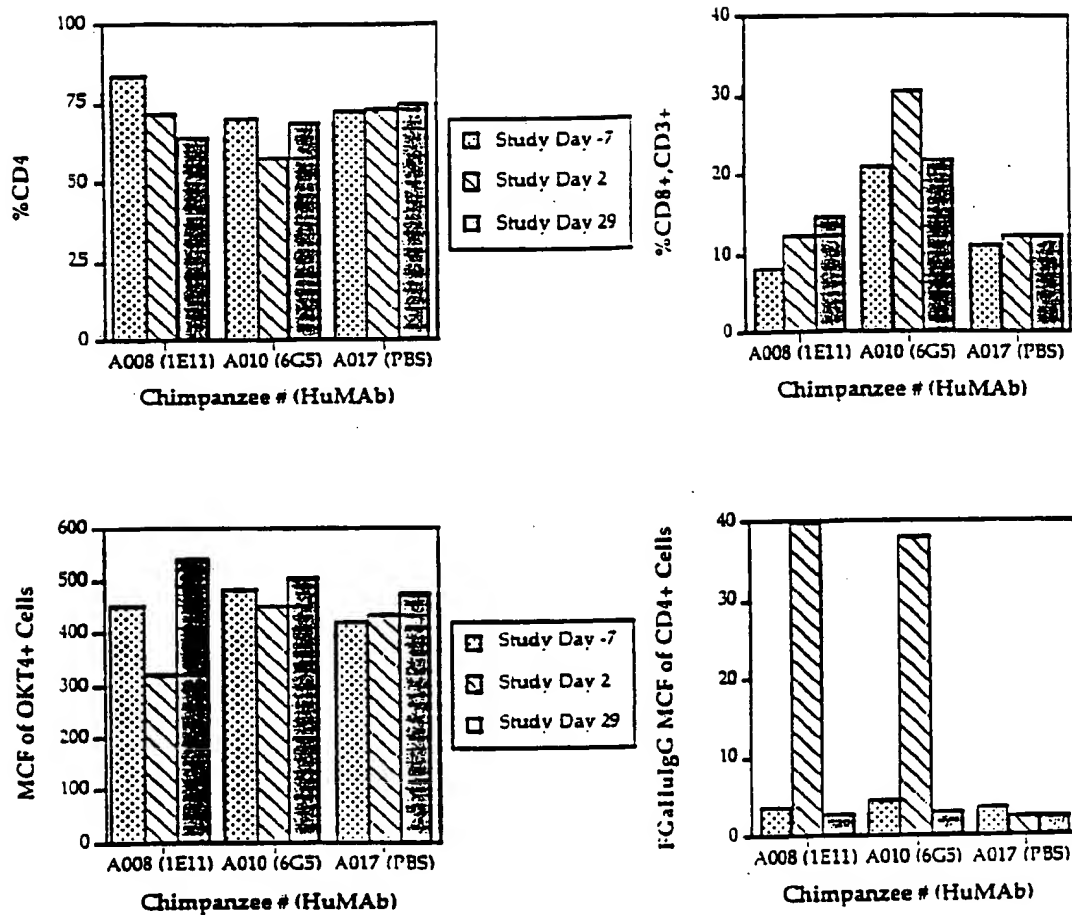
Figure 94   97/101
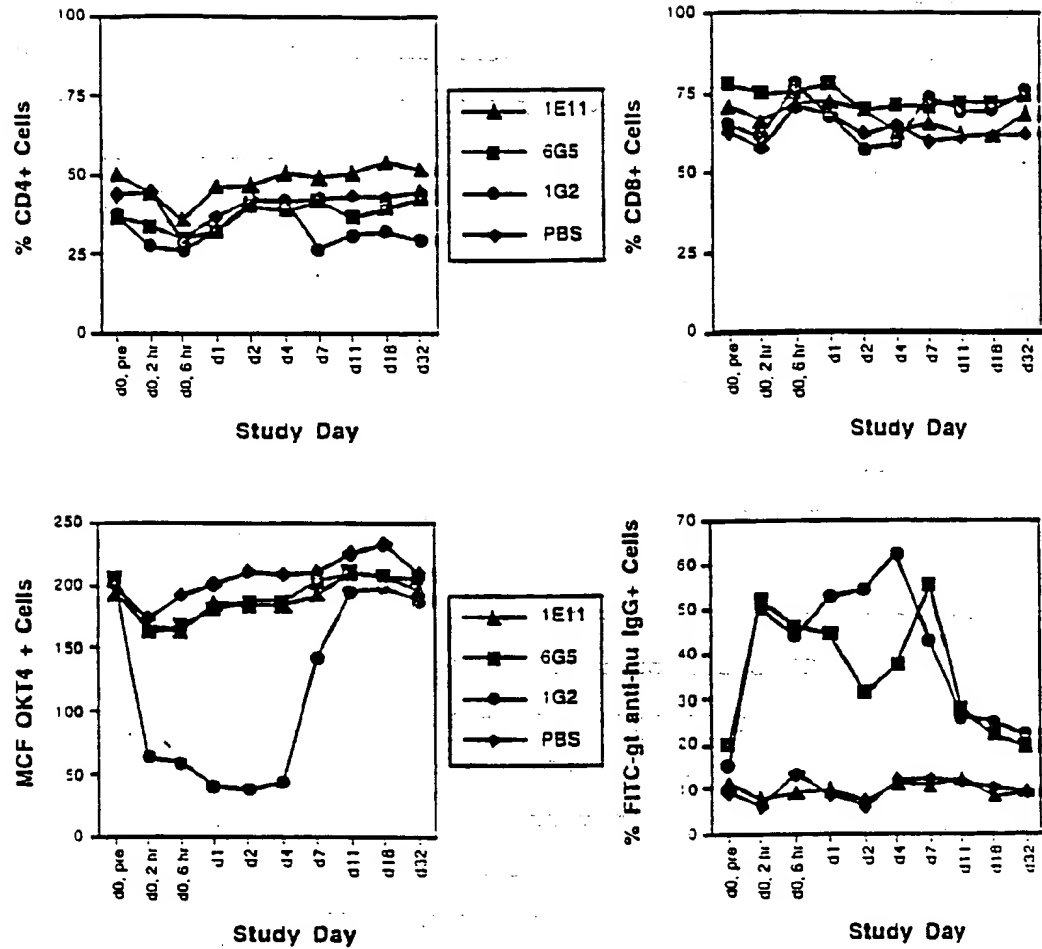
Figure 92   95/101
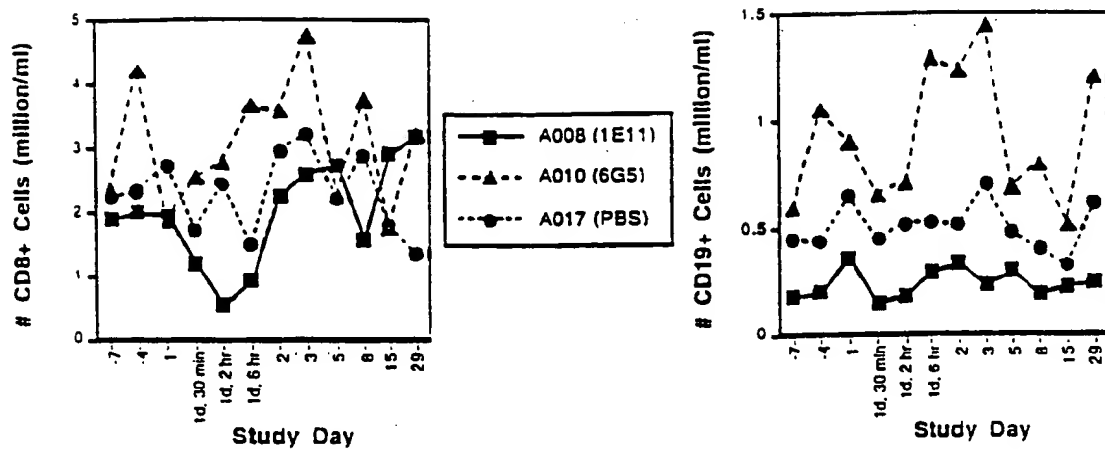
Figure 91   94/101
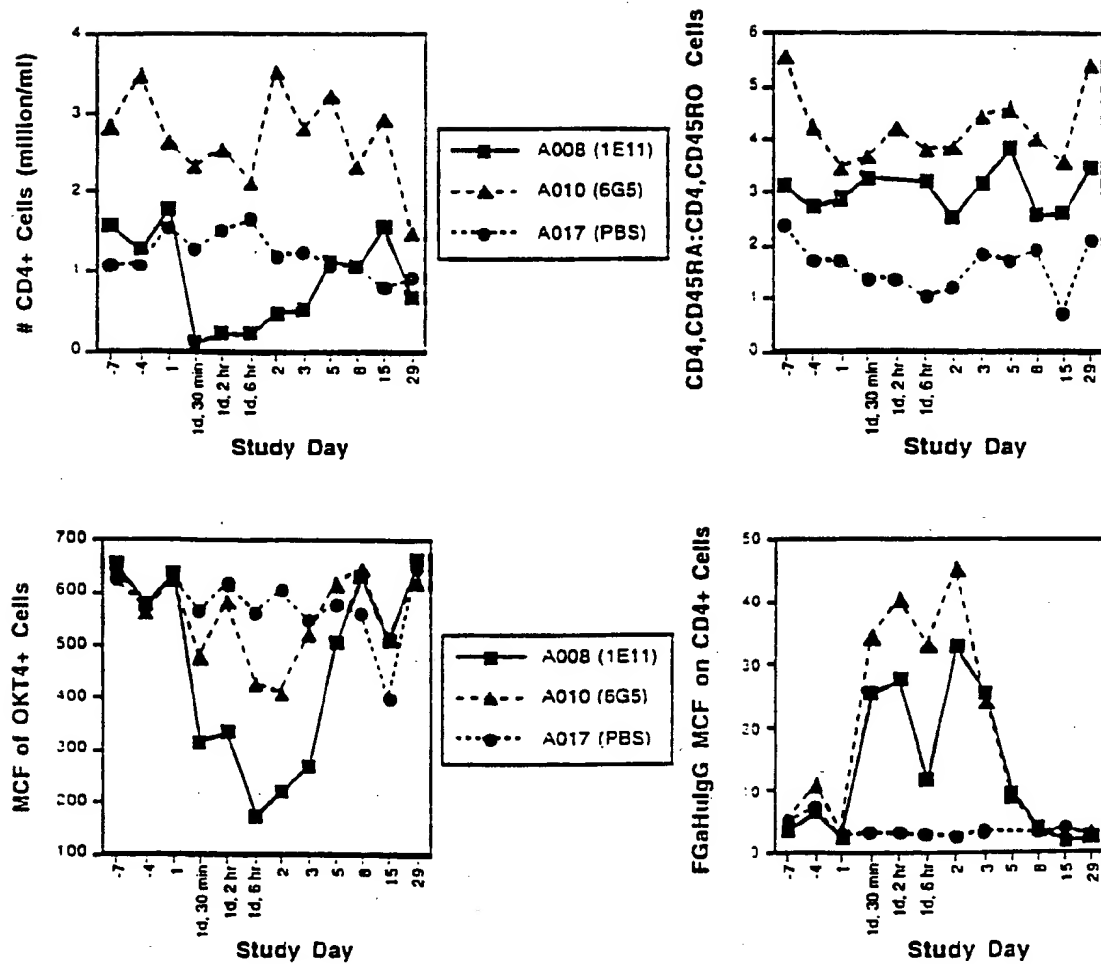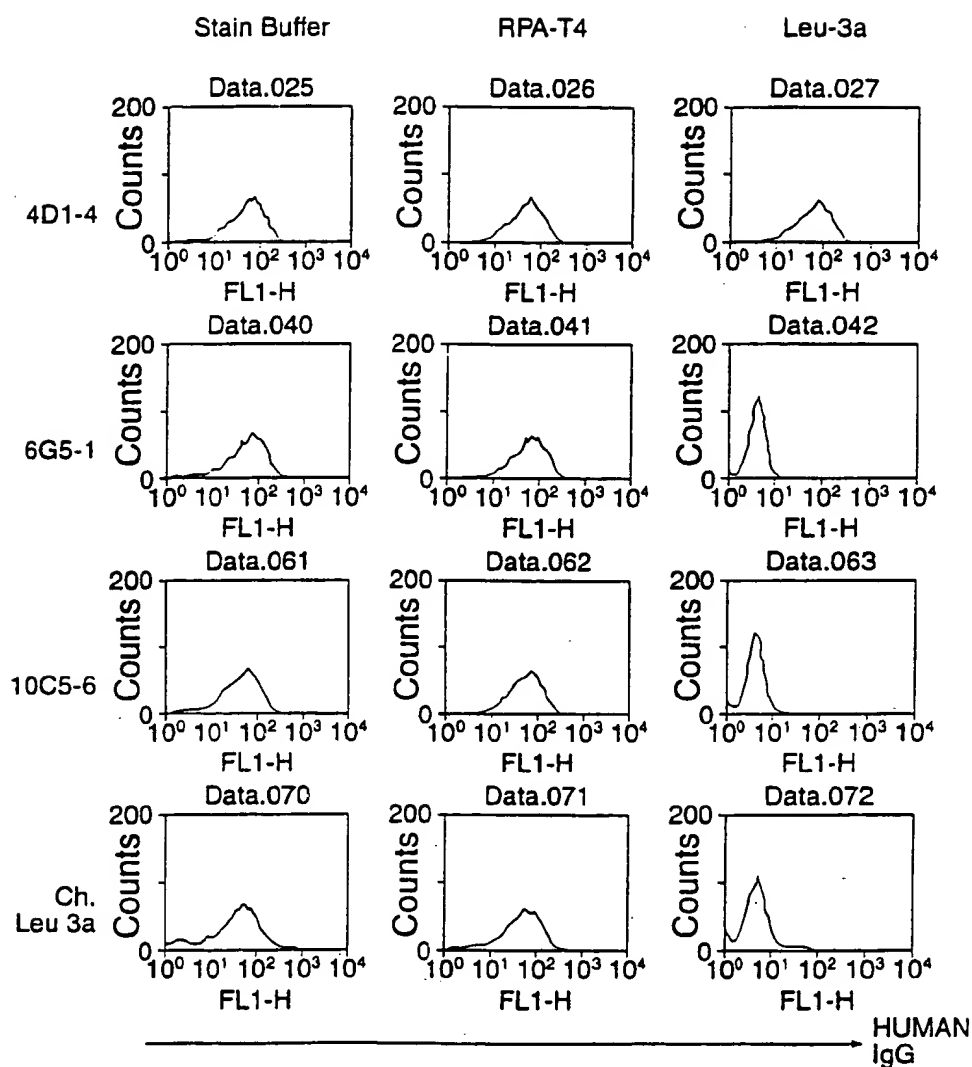
FIG. 89
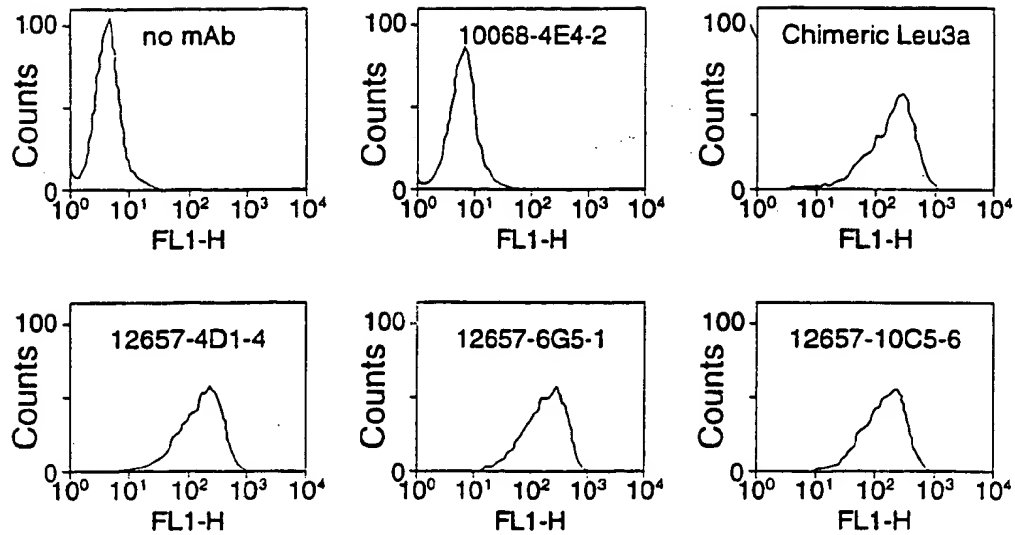
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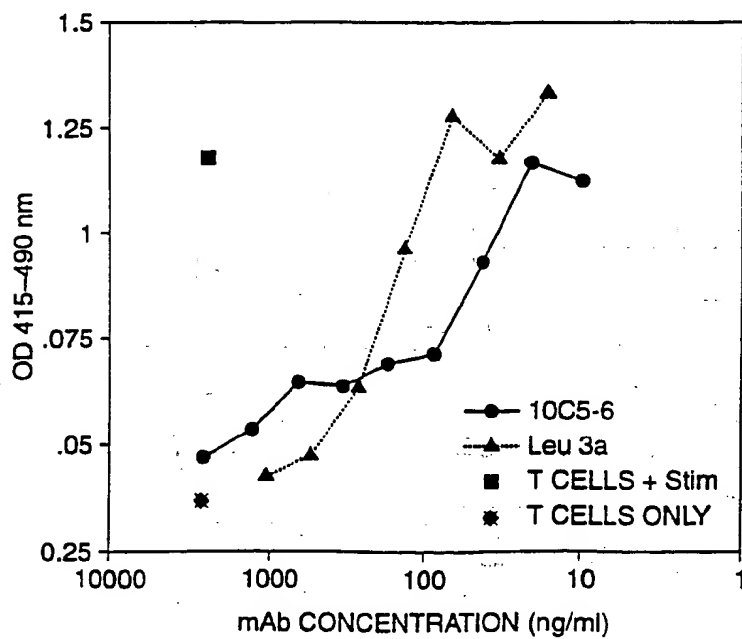
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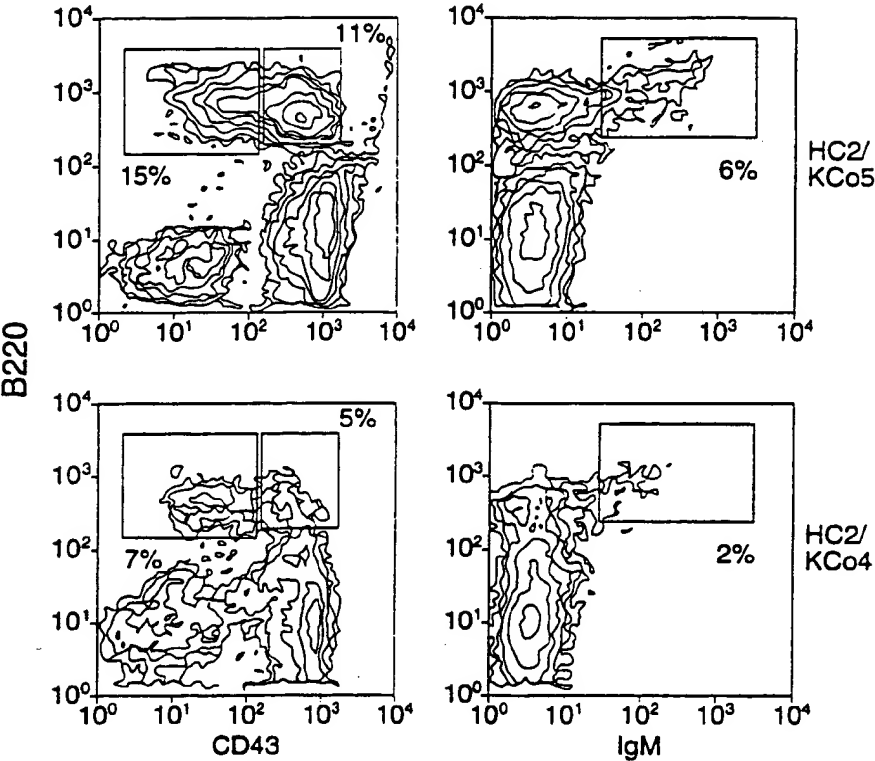
FIG. 86

FIG. 87

FIG. 85

FIG. 83

FIG. 82

FIG. 81

FIG. 84

FIG. 80

pSP72

pUC19

pGP1f

FIG. 79

FIG. 78

15 Sal I
21 HinD III
25 BstB I
28 EcoR I
37 Cla I
45 Asp718
45 Kpn I
52 BamH I
57 PaeR7 I
57 Xho I

Aat II 3047   102 Ban II
Ssp I 2931   181 Msc I
                                          184 Dsa I

                                               401 BspM II
                                               464 Eco47 III

Sca I 2607

Pvu I 2496

                    pGP2b

Pst I 2370   803 Pvu II

Ase I 2300   3122 BASE PAIRS
                     UNIQUE SITES   956 Tth111 I
Cfr10 I 2209   964 BsaA I
                                           983 Xca I
                                           1034 Nde I

                                        1212 Afl III

Sfi I 1639
Sty I 1635
BssH II 1630
Xcm I 1626
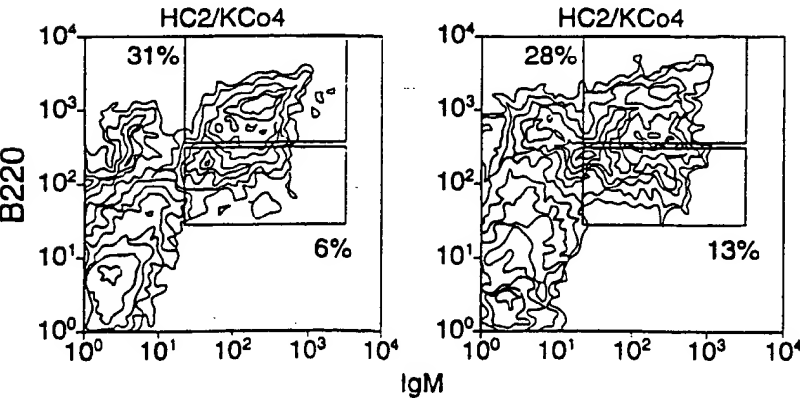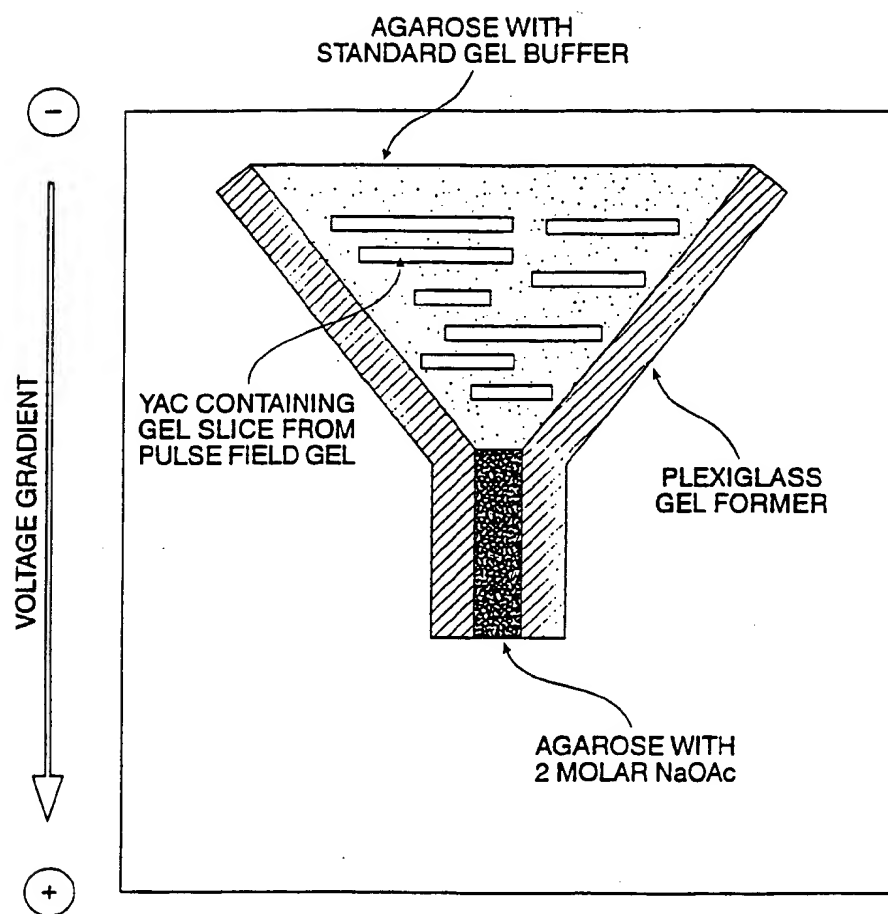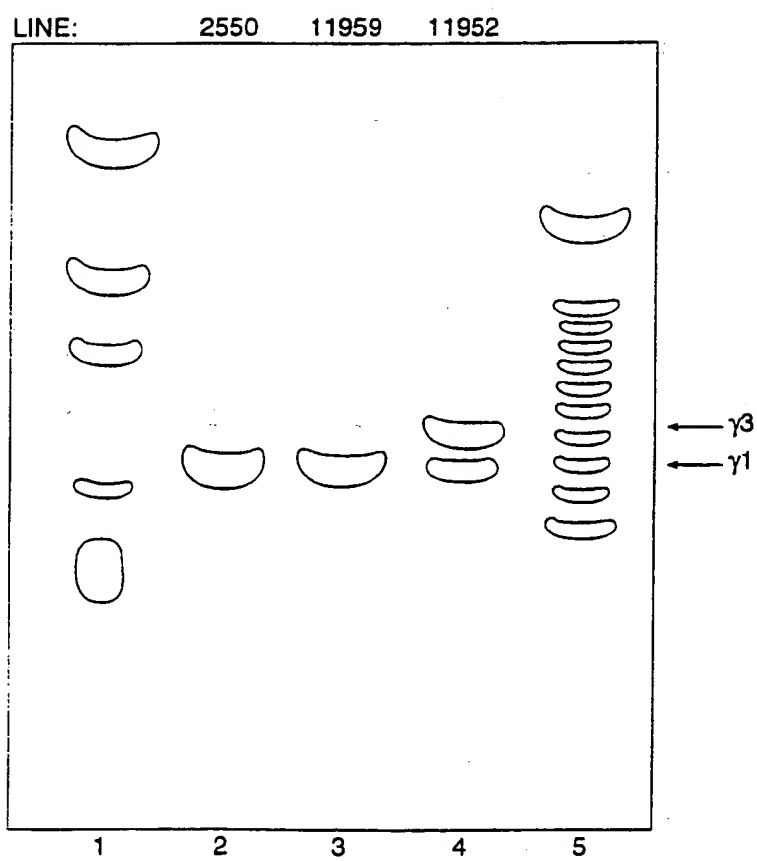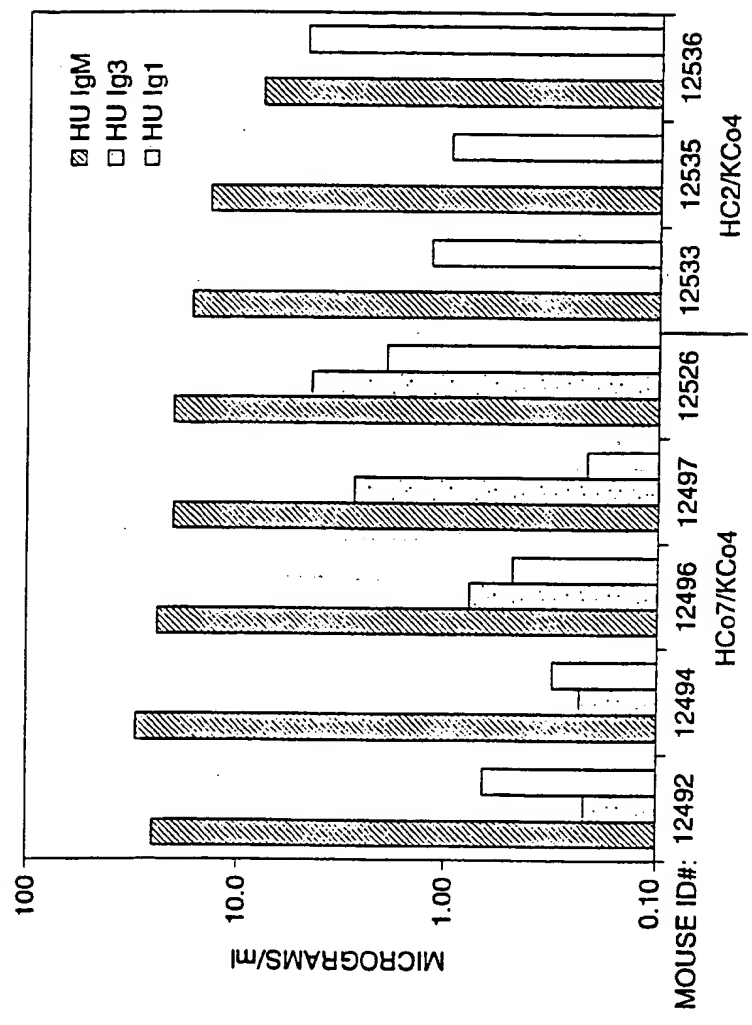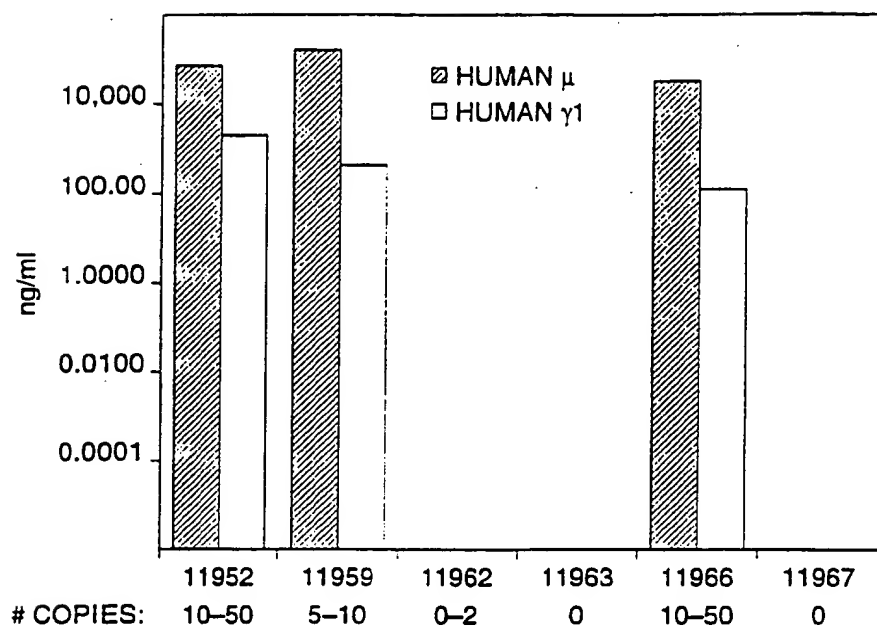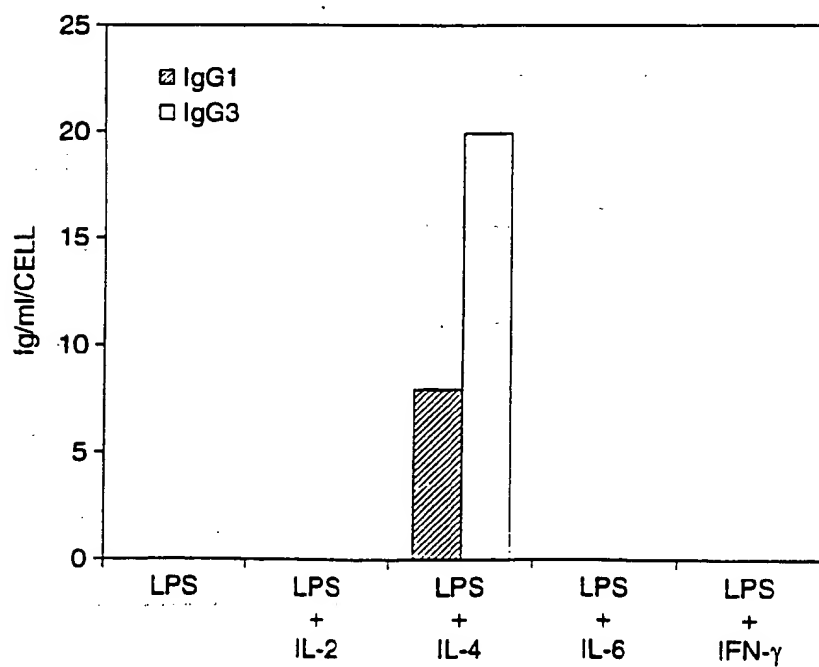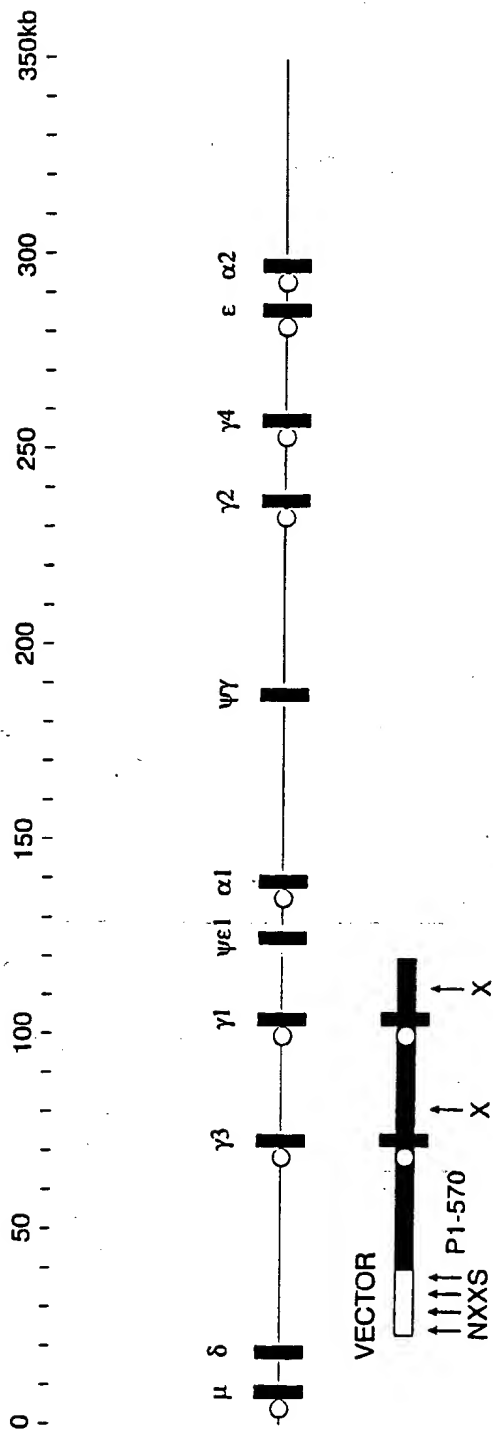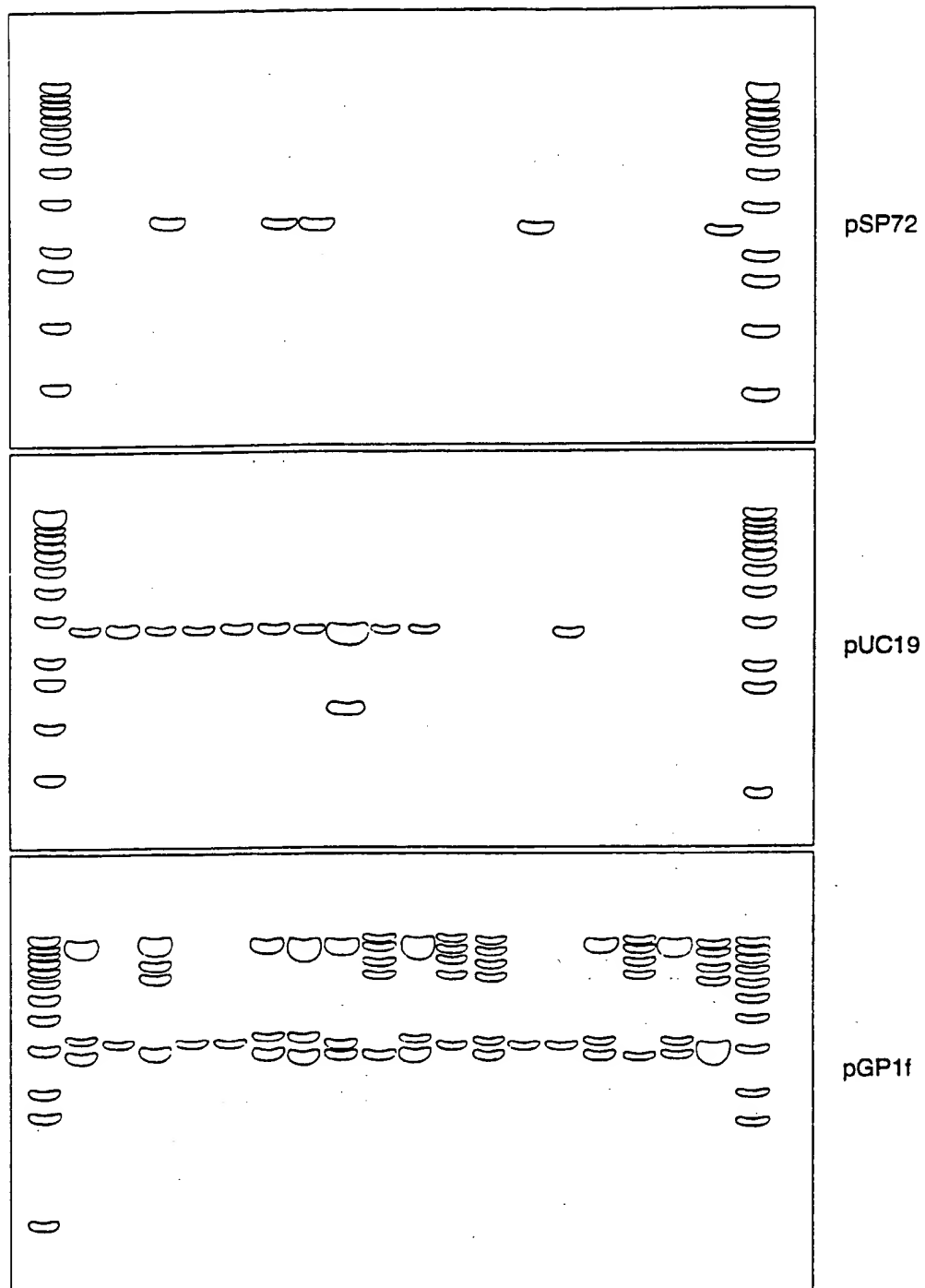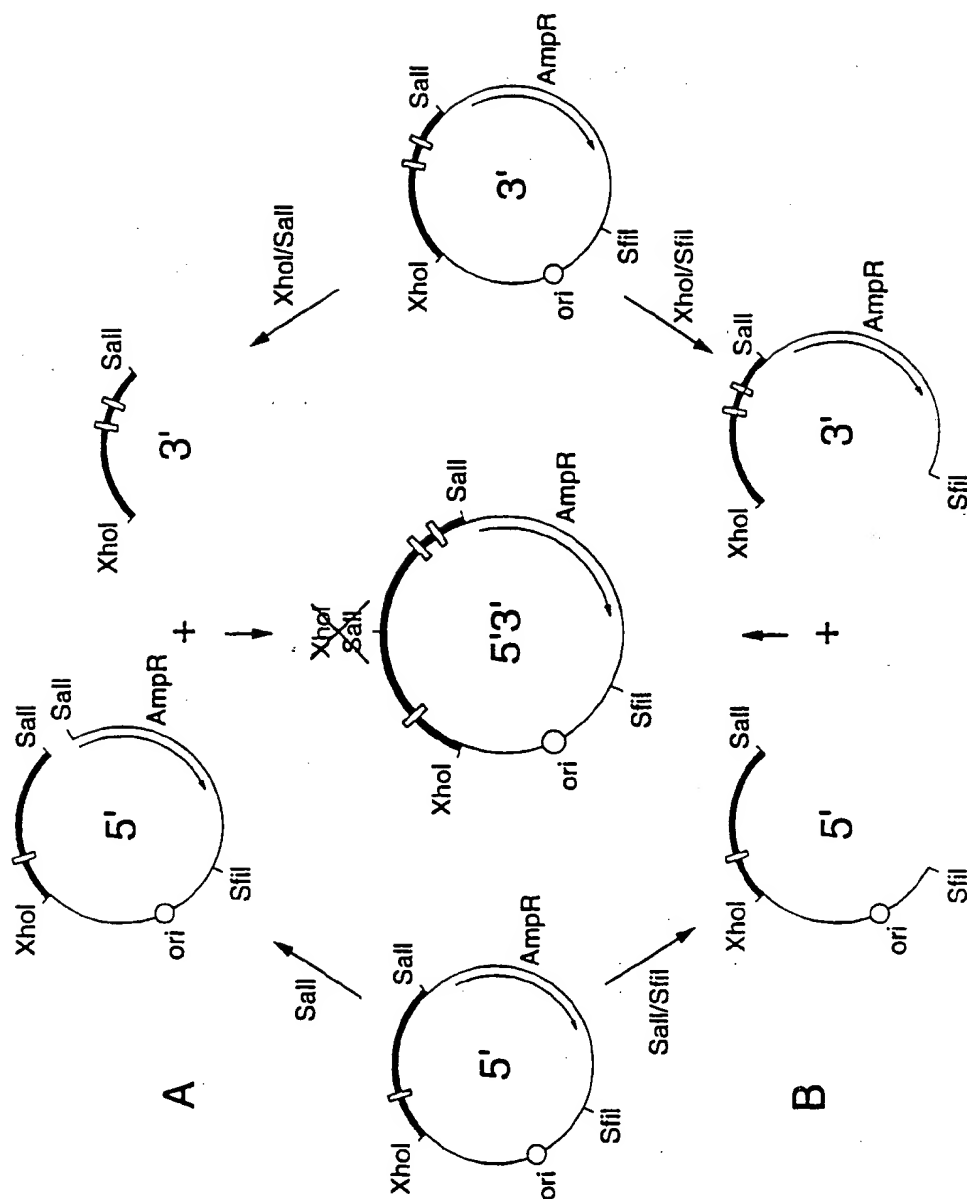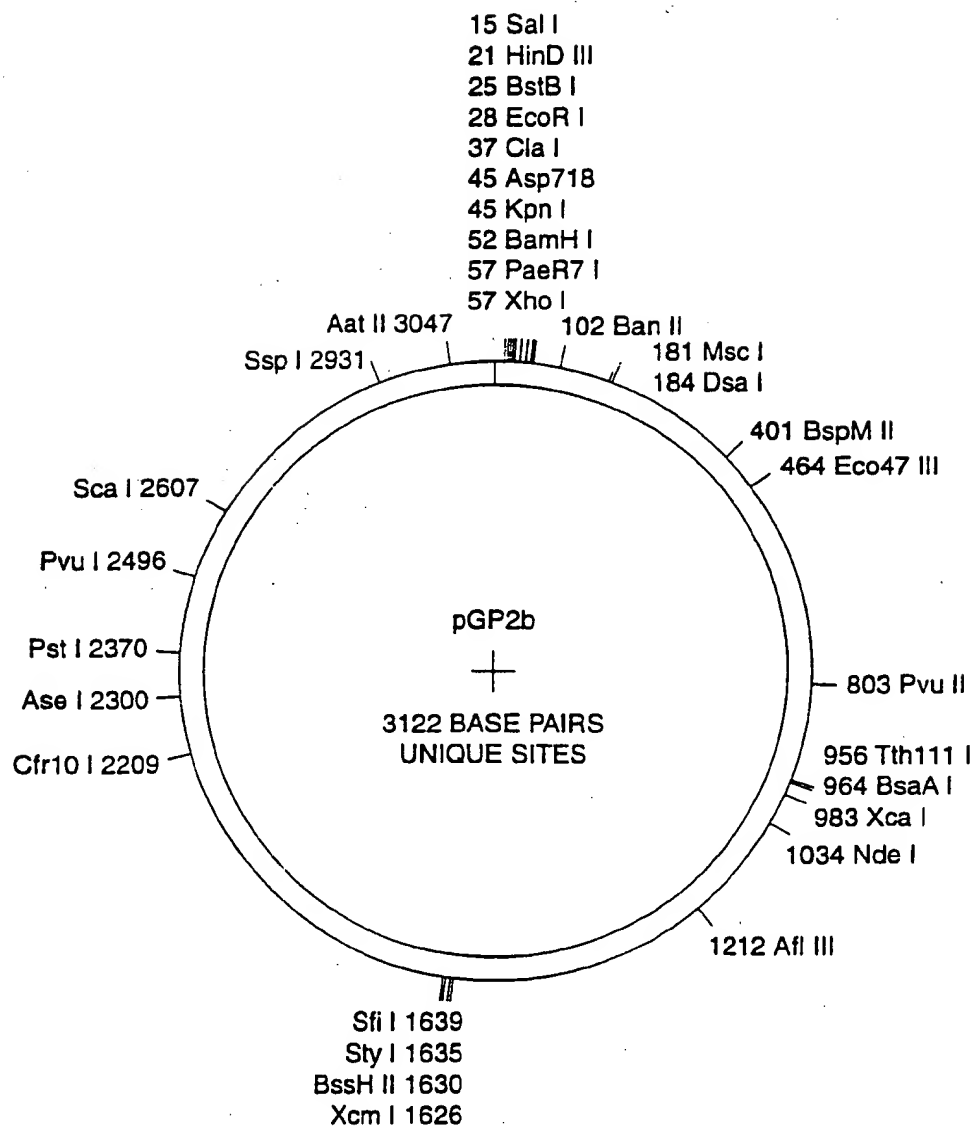
FIG. 77B

pGP2b sequence:

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AAAAAAGCCCGCTCATTAGGCGGGCTCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCA
TCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGT
TGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTG
AGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGT
TCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAAGCGCTGGCATTGACCC
TGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCCAGTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATGTTC
ATCATCAGTAACCCGTATCGTGAGCATCCTCTCTCGTTTCATCGGTATCATTACCCCCATGAACAGAAATTCCCCCTTAC
ACGGAGGCATCAAGTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTC
TGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTAC
CGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT
GTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCC
AGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGC
GGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTG
CGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAA
CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATA
GGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAC
CAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT
CCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG
GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGA
CACGACTTATCGCCACTGGCAGCAGCCAggcgcgccttggcctaagaggccaCTGGTAACAGGATTAGCAGAGCGAGGTA
TGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTC
TGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGC
TCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATT
AAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA
CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG
CTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGC
CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCT
AGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTT
TGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTA
GCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT
TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTAT
GCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCA
TTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCA
CCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAA
GGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT
GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTG
CCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCA
AG

<div align="center">*FIG. 77A*</div>
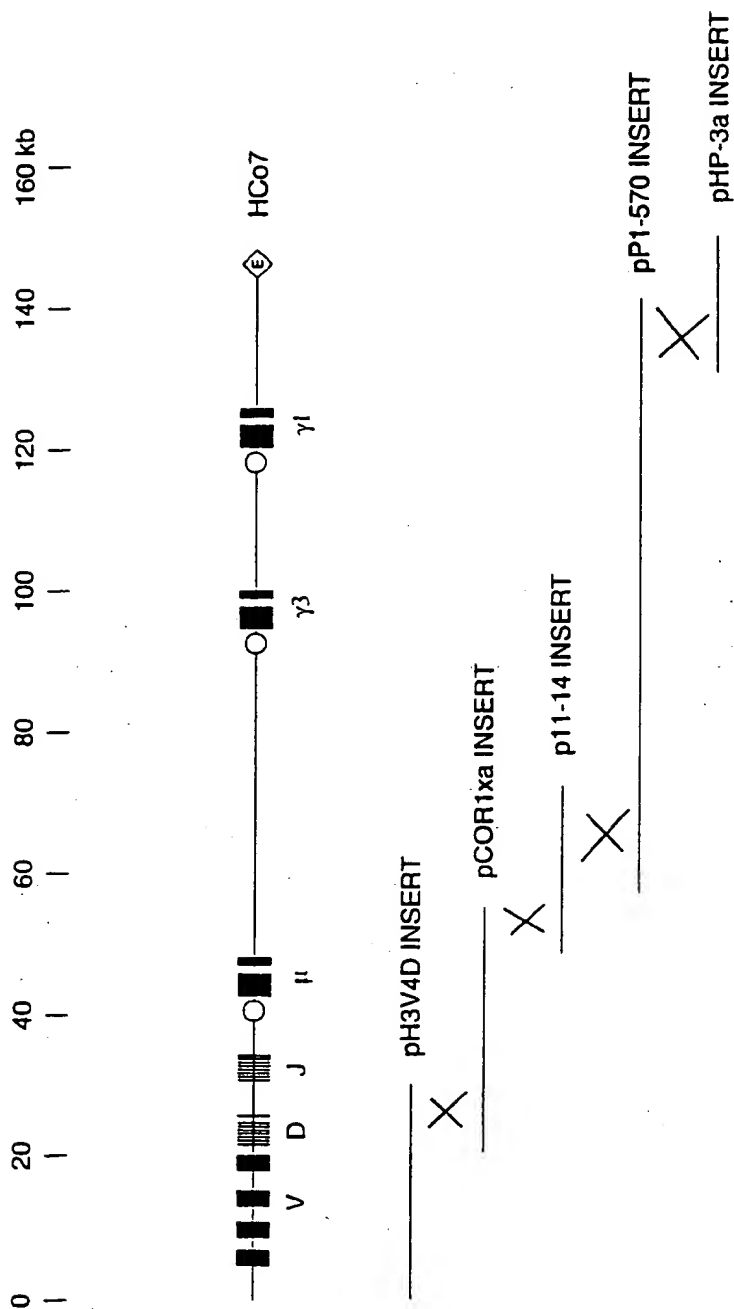
FIG. 76

RPA-T4/2C11-8
#12:BDPHARMCOMP004\FL2-H\FL2-HEIGHT

1:128

1:32

1:16

NEAT
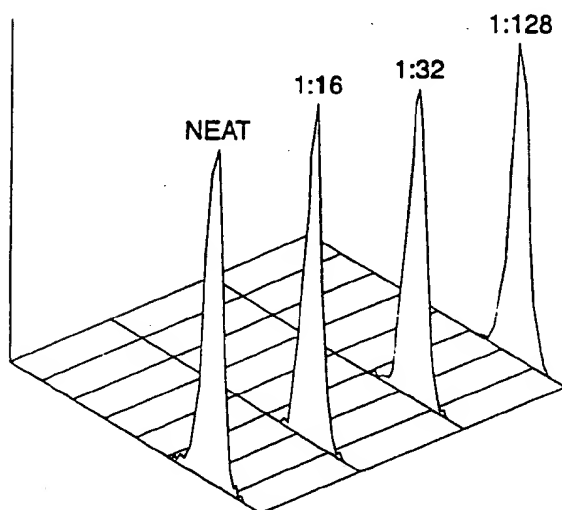
Leu-3a/2C11-8
#12:BDPHARMCOMP025\FL2-H\FL2-HEIGHT

1:128

NEAT

1:32

1:16

FIG. 75

FIG. 74

FIG. 72

FIG. 73

FIG. 71A

FIG. 71B

FIG. 69

FIG. 68B

FIG. 68A

FIG. 67B.2

FIG. 67B.1

FIG. 67A

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PCT/US97/21803

70/101

FIG. 66B.2

FIG. 66B.1

TACTGGTACTTCGATCTCTGGGGCCGTGGCAC · · · · · DHQ52

· DHQ52

· · · · · GCTTTTGATATCTGGGGCCAAGGGAC · · DHQ52

· · · · · TACTTTGACTACTGGGGCCAGGGAAC · · DHQ52

· DXP1

· DN1

· DXP'1

· DXP1

· DXP1

· DN1

TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGAC · · DXP'1

· · · · · G · · · · · · · · · · · · · · · · · DHQ52

70

ACCATCTCAGCCGACAAGTCCATCAGCAGCGACCGCCTACCTGCAGTGGAGCAGCAGCCTGAAGGCCTGGACACCGCCATGATTACTACTGTGCGAGA

80 82 a b c 83   90

CAAGGgactggggaGg · · · ·

CAGAAGGGGGGGAAGGG · · · ·

CAGAAGGGGGGGAAGGG · · · ·

actgggTGG · · · ·

CATGAaactggggaTCCGGG · · · ·

ACGGattacggtatttgactggttattataacCCT totagcagcagcCCTT · · · ·

TCTCtactatggttcggggag · · · ·

CTCGattacgatattttgactggttattataacCC · · · ·

CTCGattacgatattttgactggttattataacCC · · · ·

CCCgtatagcagcagctggtacAGG · · · ·

GAGGGGGGGTTtattactcatggttcggggagttatta · · · ·

GAGGaactggggaCCAT · · · ·

CDR III

FIG. 66A.2

VH251

HUMAN
n D n

MOUSE
Cγ

5 DXP'1 J6 G1
7 DHQ52 J3 G1

2 DHQS2 J3 G2b
3 D? J3 G2b
4 DXP'1 J4 G2b
10 DHQS2 J3 G2b

1 D? J3 G3
6 DHQS2 J4 G3
8 DIR2 J3 G3
9 DIR2r J6 G3

FIG. 47

FIG. 46

FIG. 45

FIG. 66A.1

C_γ

mouse γ1

ggtGtgAacgtctgggcaagggccaccgtctcctcag ccaaaacgacaccccatcgtgtctatccac

gctCttgatGtctggggcaagggacaatgtCtcacgtctcttcag ccaaaacgacaccccatcgtctatccac

mouse γ2b

gcttttgtatctggggccaagggacaatggtcaccgtctcttcag ccaaaacaacacccccatcagtctatccac

gctttagatatctggggccaagggacaatggtcaccgtctcttcag ccaaaacaacacccccatcagtctatccac

tgactactgggggccaggggaaccctggtcaccgtctcctcag ccaaaacaacaccccatcagtctatccac

gcttttgtatctggggccaagggacaatggtcaccgtctcttcag ccaaaacaacacccccatcagtctatccac

mouse γ3

gatatctggggccaagggacaatggtcaccgtctcttcag ctacaacaacagccctctcctcag

tttgactaTtggggccaggggaacctggtcaccgtctcctcag ctacaacaacagccctctcctcag

gcttttgtatctggggccaagggacaatggtcaccgtctcttcag ctacaacaacagccctctcctcag

tactactactacggtatggacgtctggggccaagggacaccgtctcctcag ctacaacaacagccctctcctcag

mouse

J

V_H251   N D N

2357.t5 DXP'1 J6
gcctcggacaccgccatgtattactgtgtgaga CATTtatggttcggggagttaCG

2357.t7 DHQ52 J3
gcctcggacaccgccatgtattactgtgtgcgaga CactgggCATTGGAT

2357.t2 DHQ52 J3
gcctcggacaccgccatgtattactgtgtgcgaga actggggaTGAT

2357.t3 D? J3
gActcggacaccgccatgtattactgtgtgcgaga CAGGGGAGAGAT

2357.t4 DXP'1 J4
gcctcggacaccgccatgtattaTtgtgtgaga CATAGGGactataTttcggggagttattTCC

2357.t10 DHQ52 J3
gcctcggacaccgccatgtattactgtgtgcgaga actggggaTGAT

2357.t1 D? J3
gcctcggacaccgccatgtattactgtgtgcgaga CATGGGTCTATG

2357.t6 DHQ52 J4
gcctcggacaccgccatgtattactgtgtgcgaga GAGACGGTCactggggaTCG

2357.t8 DIR2 J3
gcctcggacaccgccatgtattactgtgtgcgaga AGGgaccccCTGAT

2357.t9 DIR2R J6
gcctcggacaccgccatgtattactgtgtgcgaga CGggggcct

human

FIG. 65

FIG. 64B

FIG. 64A

FIG. 63

FIG. 70

FIG. 62

CGAGAGGGGCGGGGGGAAGACTACTATCCCAGGCAGGTTTTAGGTTCCAGAGTCTGCGAG
AAATCCCACCATCTACCCACTGACACTCCCACCAGTCCTGTGCAGTGATCCCGTGATAAT
CGGCTGCCTGATTCACGATTACTTCCCTTTCGGCACGATGAATGTGACCTGGGGAAAGAG
TGGGAAGGATATAACCACCGTGAACTTTCCACCTGCCCTCGCCTCTGGGGGACGGTACAC
CATGAGCAGCCAGTTAACCCTGCCAGCTGTCGAGTGCCCAGAAGGAGAGTCCGTGAAATG
TTCCGTGCAACATGACTCTAACCCCGTCCAAGAATTGGATGTGAATTGCTCTGGTAAAGA
ACGTTAGGGGGTCAGCTAGGGGTGGGATAAGTCCTACCTTATCTAGATCCATATATCCCT
CTGATGCACACCCTCACAGGAATCCCTCAGAAACCTCCACTATGGGGATTGGGGGAAGGA
AGCGTAAACAGGTCTAGAAGGAGCTGGAGGCCTCAGAACATCCAGAAACGGGGACAGCAA
AGGAGACAAGGAGAATATACTGATTTGCTAGGACATCTTCTGTTACAGGTCCTACTCCTC
CTCCTCCTATTACTATTCCTTCCTGCCAGCCCAGCCTGTCACTGCAGCGGCCAGCTCTTG
AGGACCTGCTCCTGGGTTCAGATGCCAGCATCACATGTACTCTGAATGGCCTGAGAAATC
CTGAGGGAGCTGCTTTCACCTGGGAGCCCTCCACTGGGAAGGATGCAGTGCAGAAGAAAG
CTGCGCAGAATTCCTGCGGCTGCTACAGTGTGTCCAGCGTCCTGCCTGGCTGTGCTGAGC
GCTGGAACAGTGGCGCATCATTCAAGTGCACAGTTACCCATCCTGAGTCTGGCACCTTAA
CTGGCACAATTGCCAAAGTCACAGGTGAGCTCAGATGCATACCAGGACATTGTATGACGT
TCCCTGCTCACATGCCTGCTTTCTTCCTATAATACAGATGCTCAACTAACTGCTCATGTC
CTTATATCACAGAGGGAAATTGGAGCTATCTGAGGAACTGCCCAGAAGGGAAGGGCAGAG
GGGTCTTGCTCTCCTTGTCTGAGCCATAACTCTTCTTTCTACCTTCCAGTGAACACCTTC
CCACCCCAGGTCCACCTGCTACCGCCGCCGTCGGAGGAGCTGGCCCTGAATGAGCTCTTG
TCCCTGACATGCCTGGTGCGAGCTTTCAACCCTAAAGAAGTGCTGGTGCGATGGCTGCAT
GGAAATGAGGAGCTGTCCCCAGAAAGCTACCTAGTGTTTGAGCCCCTAAAGGAGCCAGGC
GAGGGAGCCACCACCTACCTGGTGACAAGCGTGTTGCGTGTATCAGCTGAAACCTGGAAA
CAGGGTGACCAGTACTCCTGCATGGTGGGCCACGAGGCCTTGCCCATGAACTTCACCCAG
AAGACCATCGACCGTCTGTCGGGTAAACCCACCAATGTCAGCGTGTCTGTGATCATGTCA
GAGGGAGATGGCATCTGCTACTGAGCCACCCTGCCTGTCCCTACTCCTAGAATAAACTCT
GTGCTCATCCAAAGTATCCCTGCACTTCCACCCAGTGCCTGTCCACCACCCTGGGGTCTA
CGAAACACAGGGAGGGGTCAGGGCCCAGGGAGGGAGAAATACCACCACCTAAGC

<div align="center">

FIG. 61

</div>
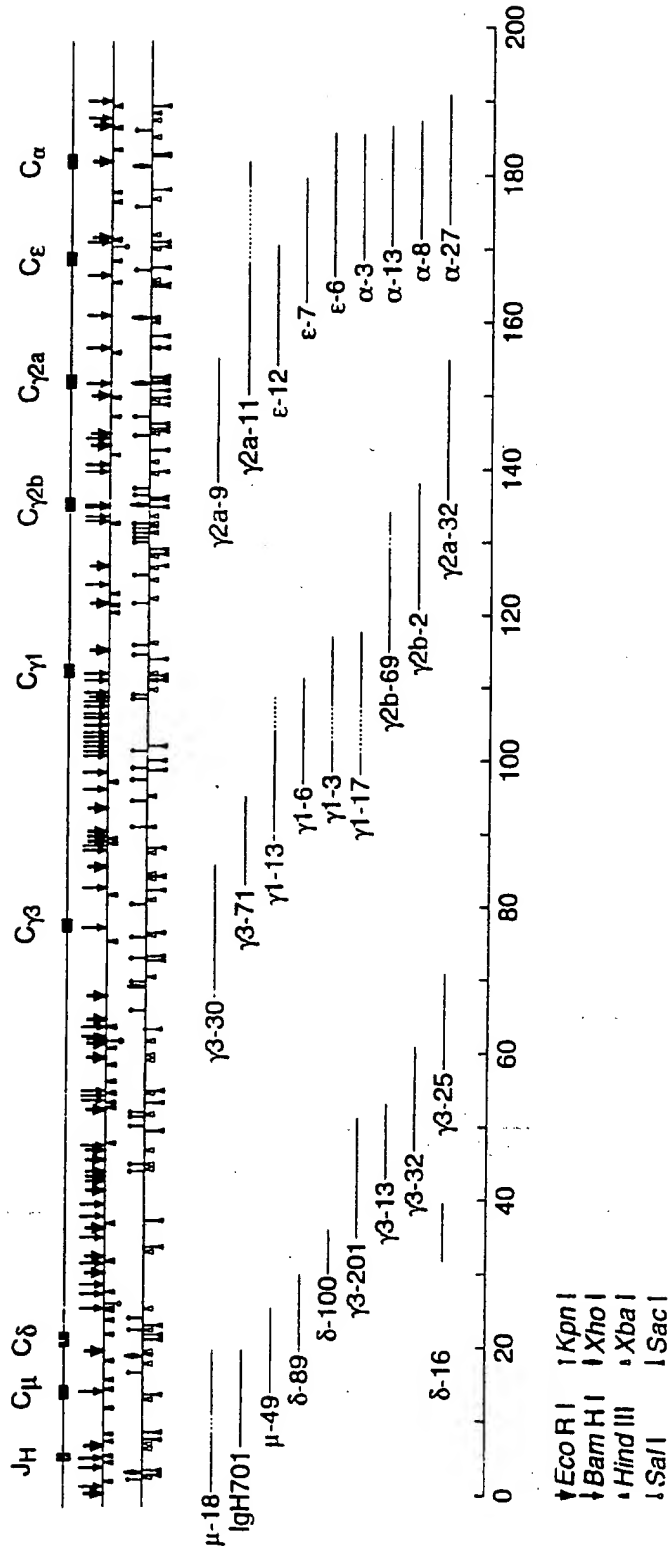
FIG. 60
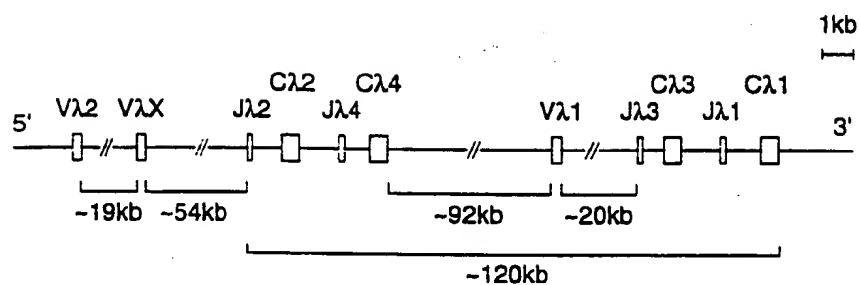
FIG. 57

GENERATION OF A SINGLE LARGE DELETION
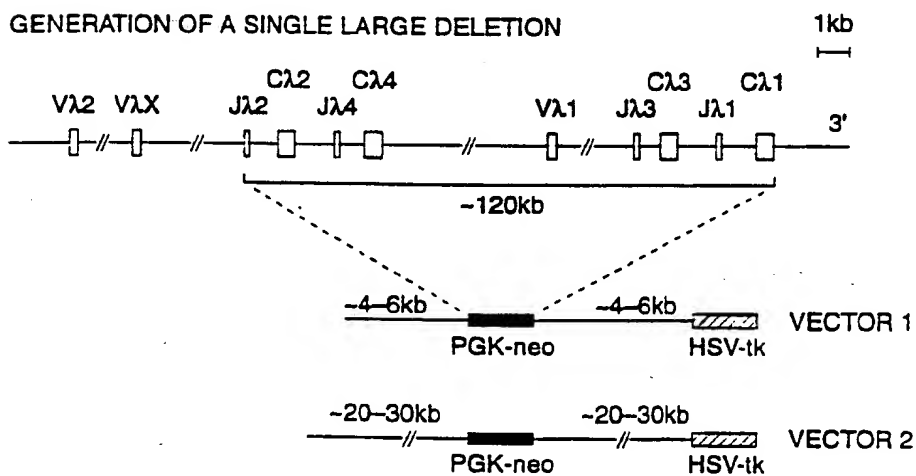
GENERATION OF TWO SMALL DELETIONS
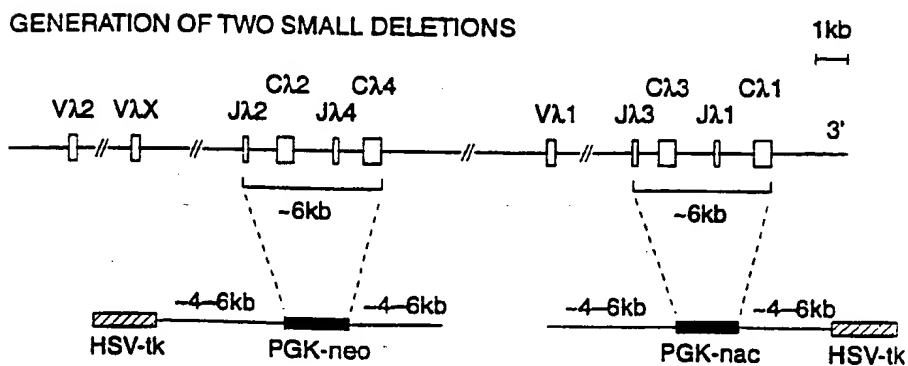
FIG. 58
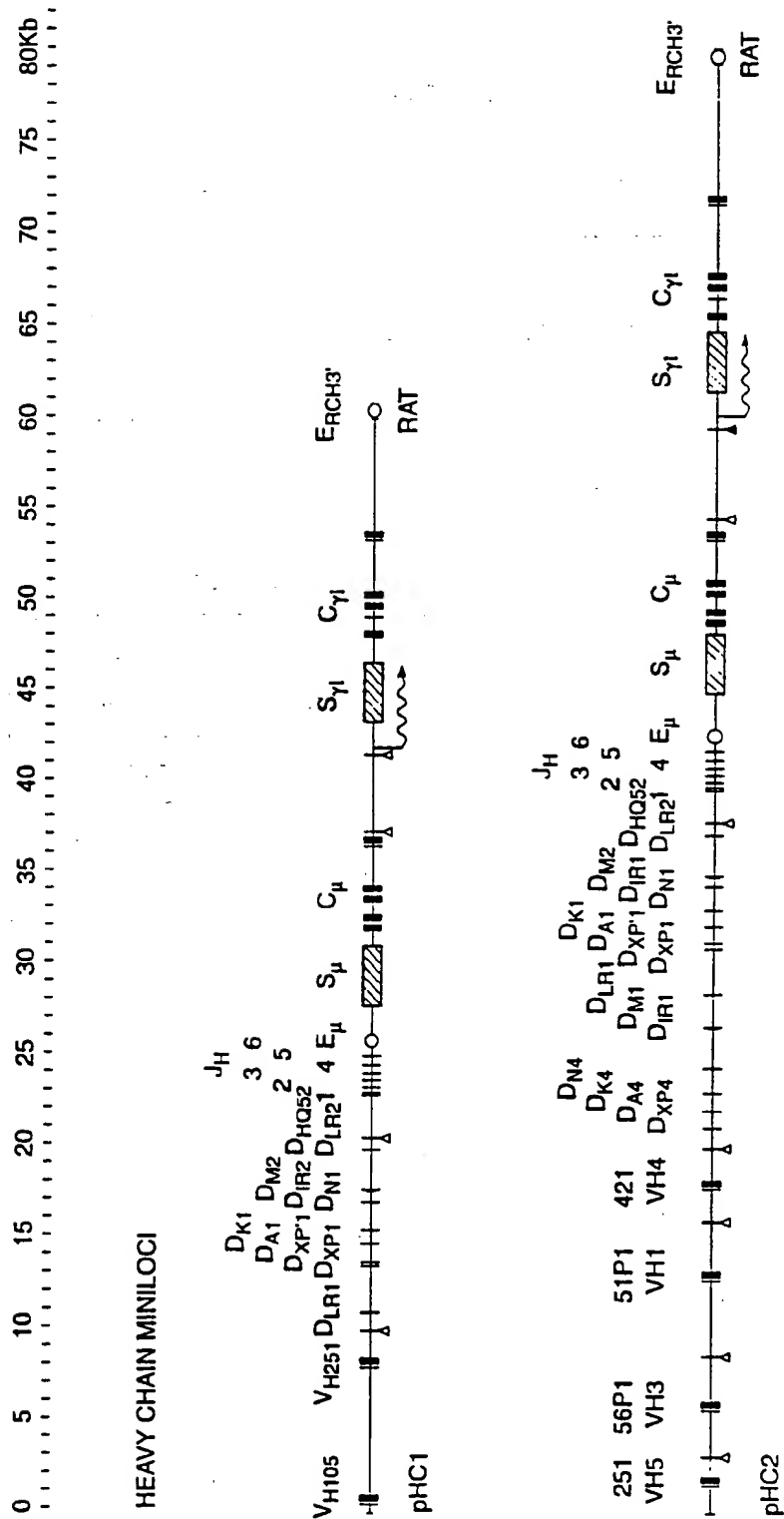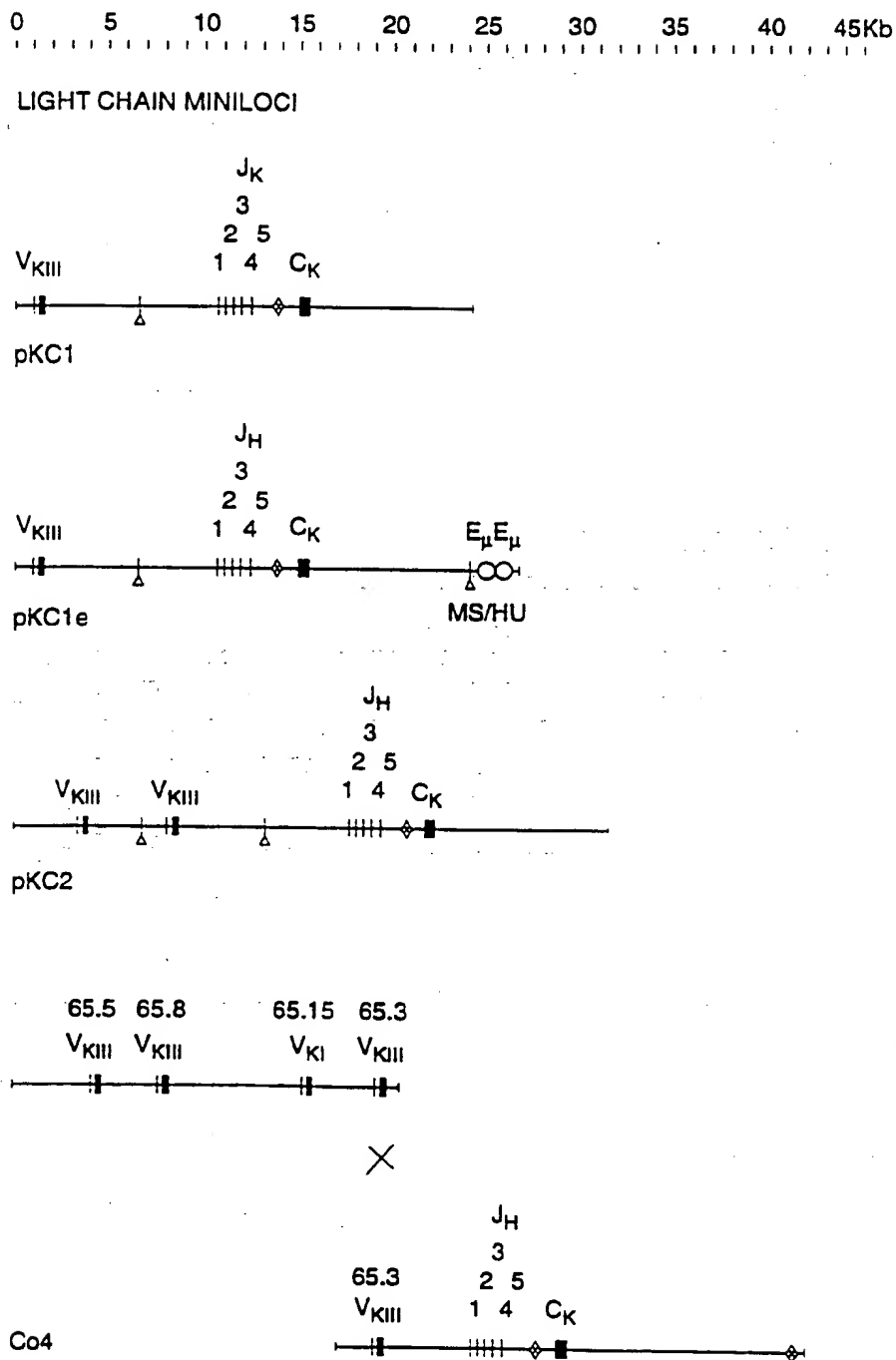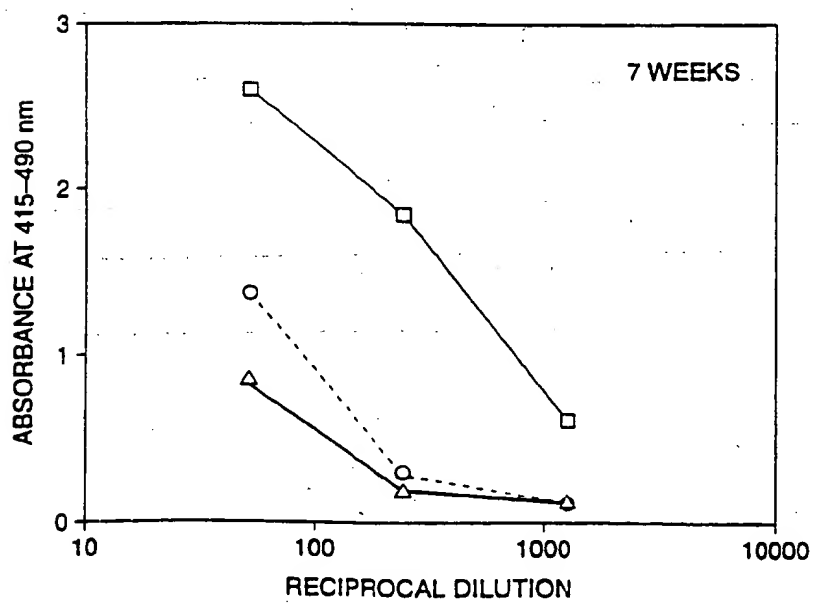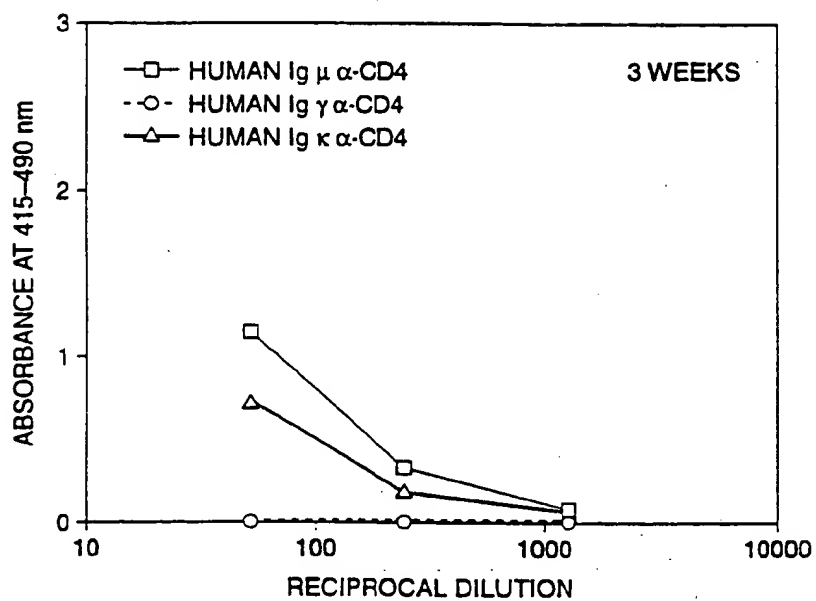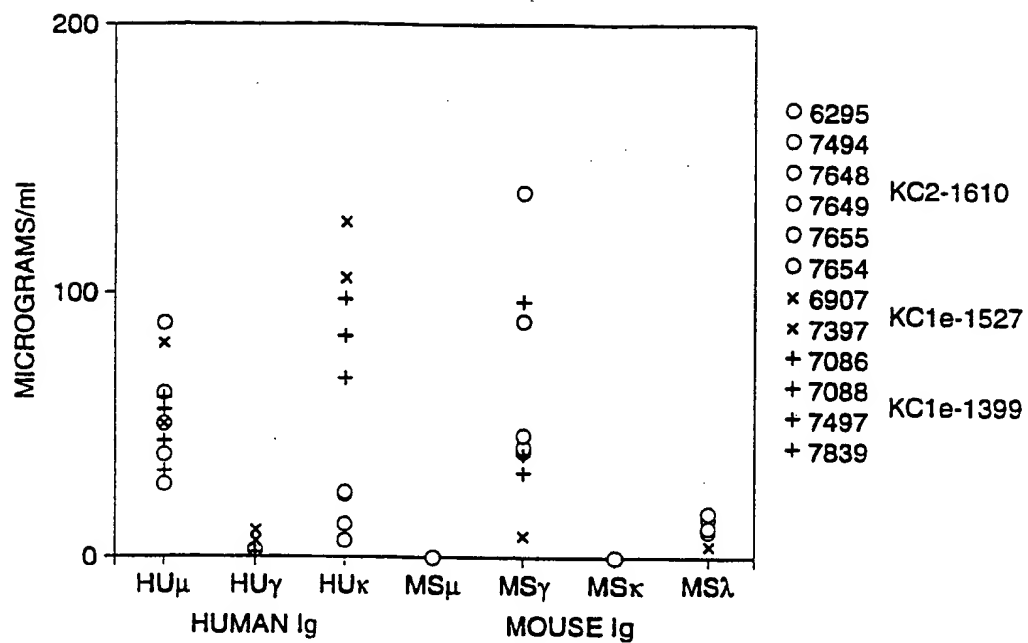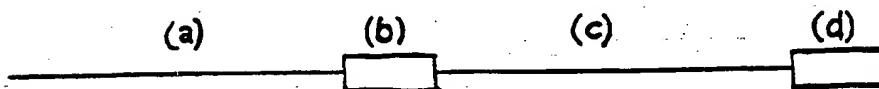
FIG. 56B

FIG. 56A

FIG. 55

FIG. 54

FIGURE 59
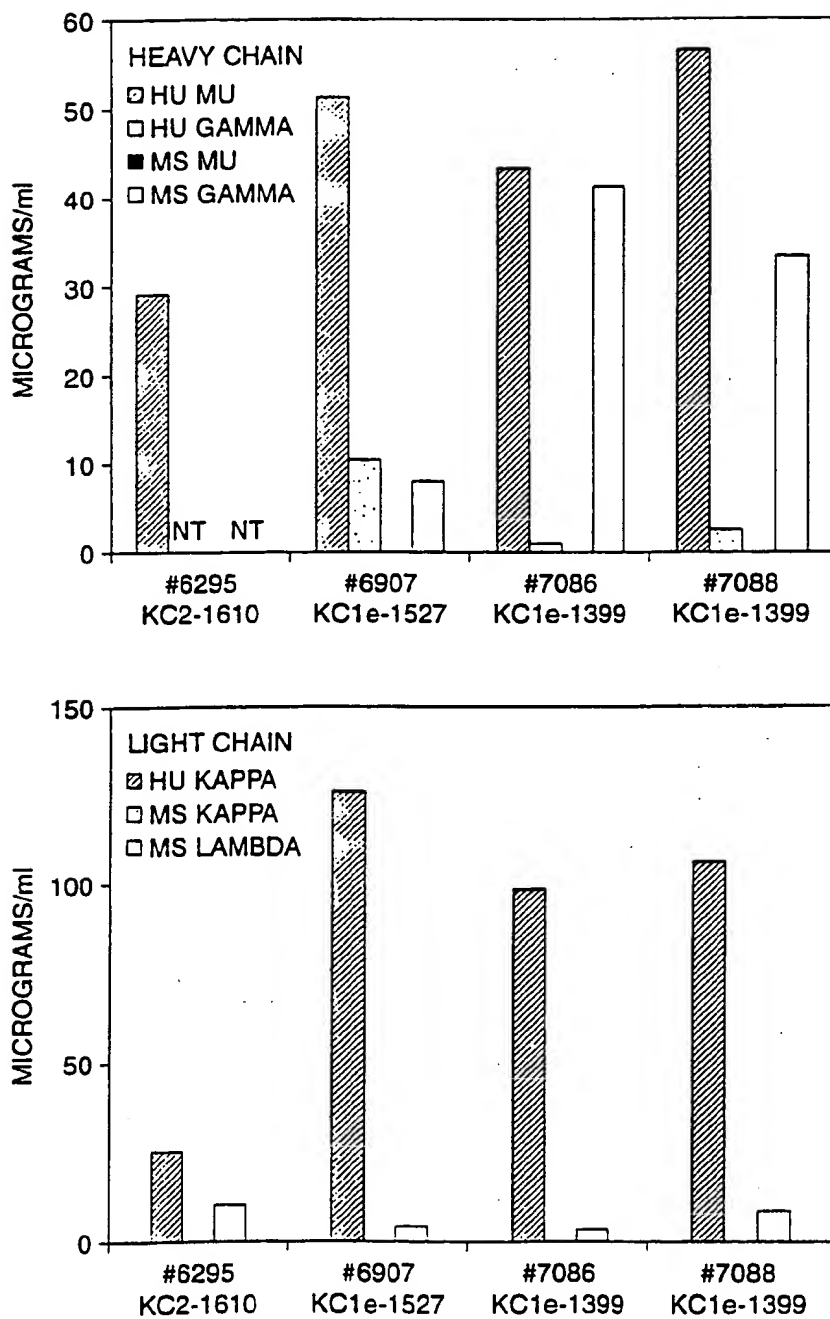
FIG. 53
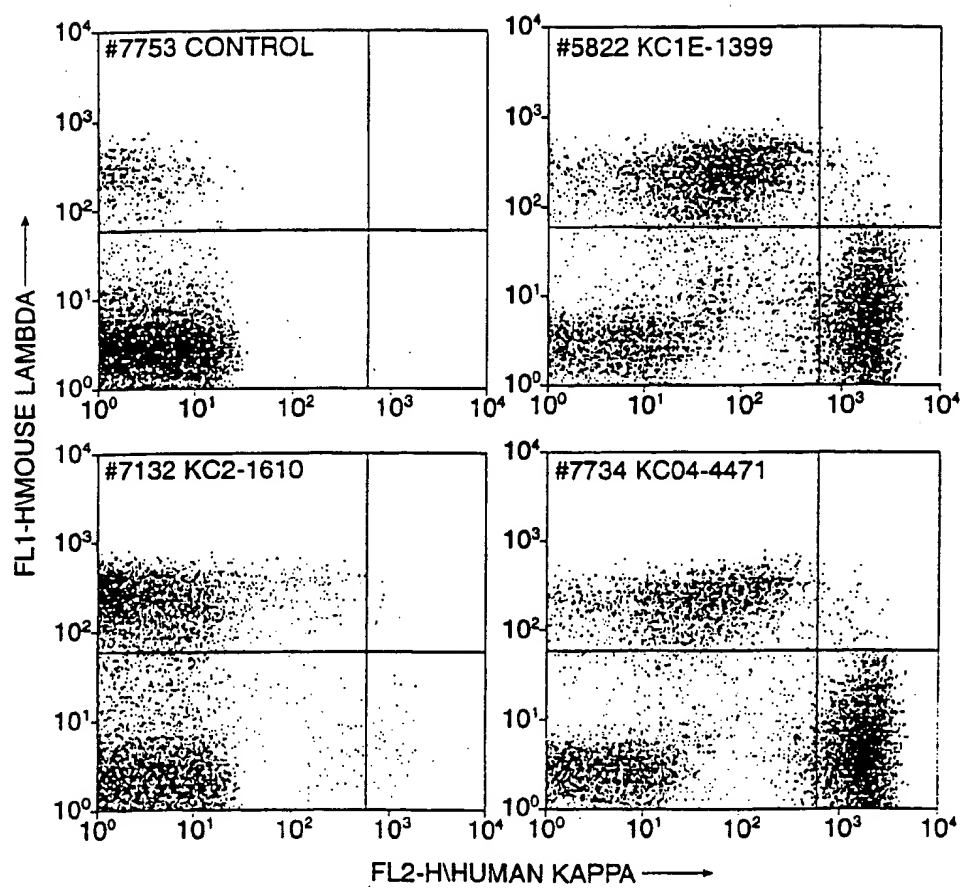
FIG. 52
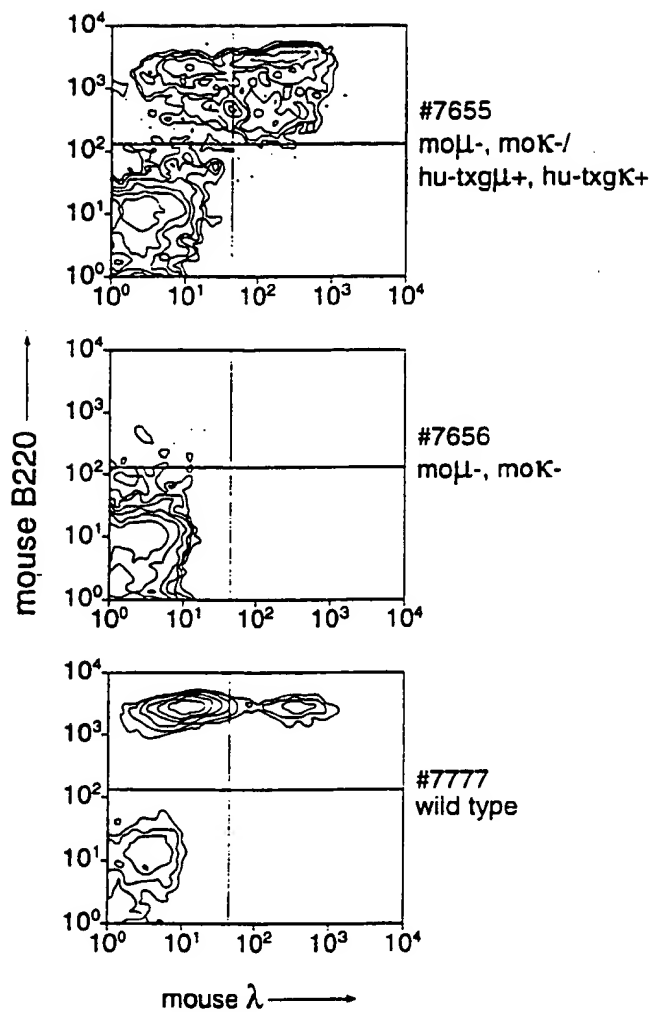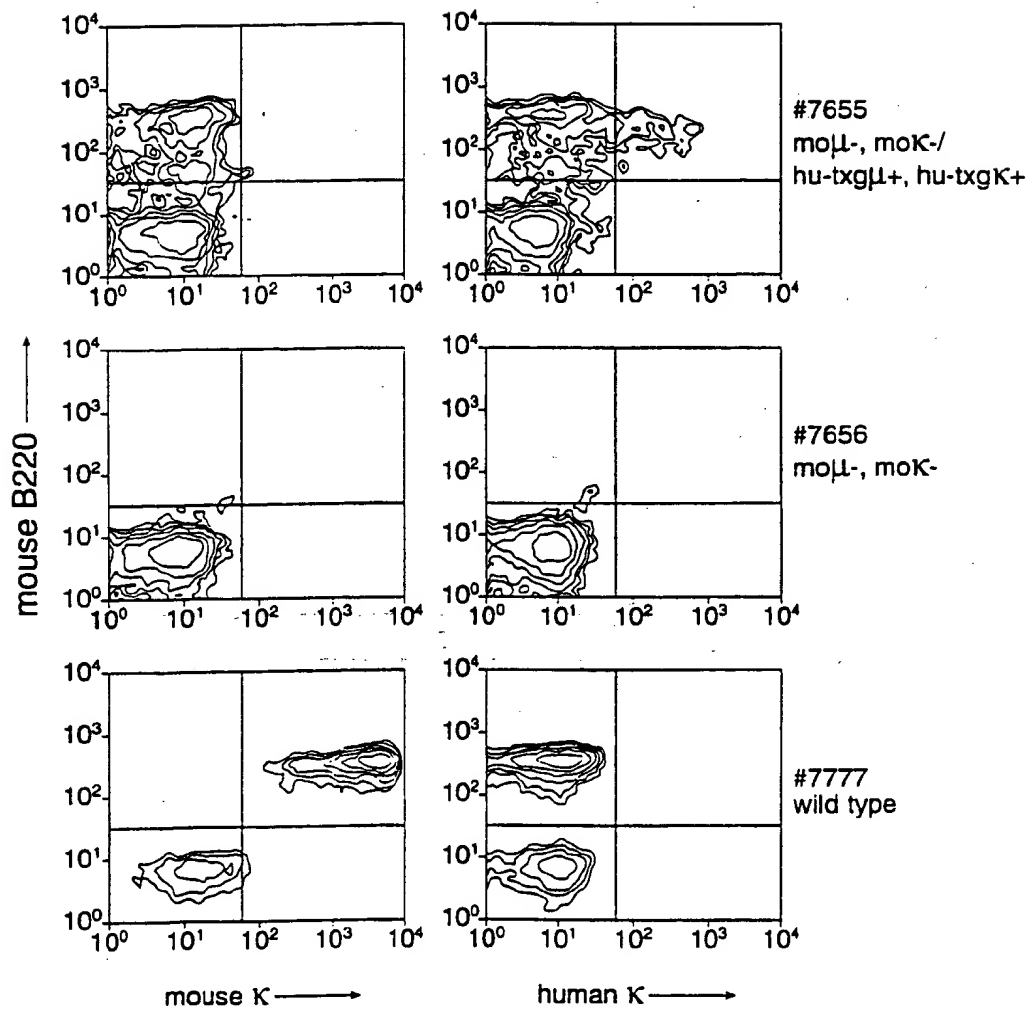
FIG. 51

FIG. 50
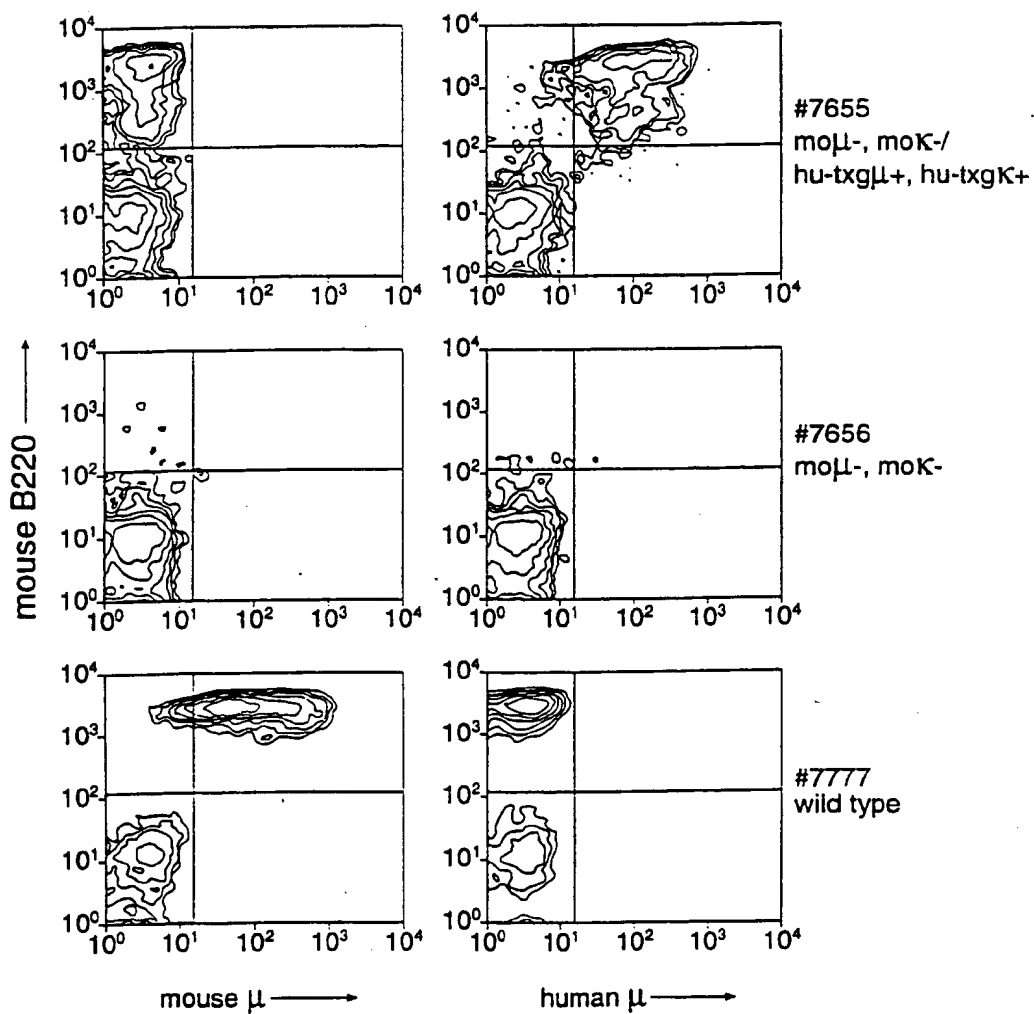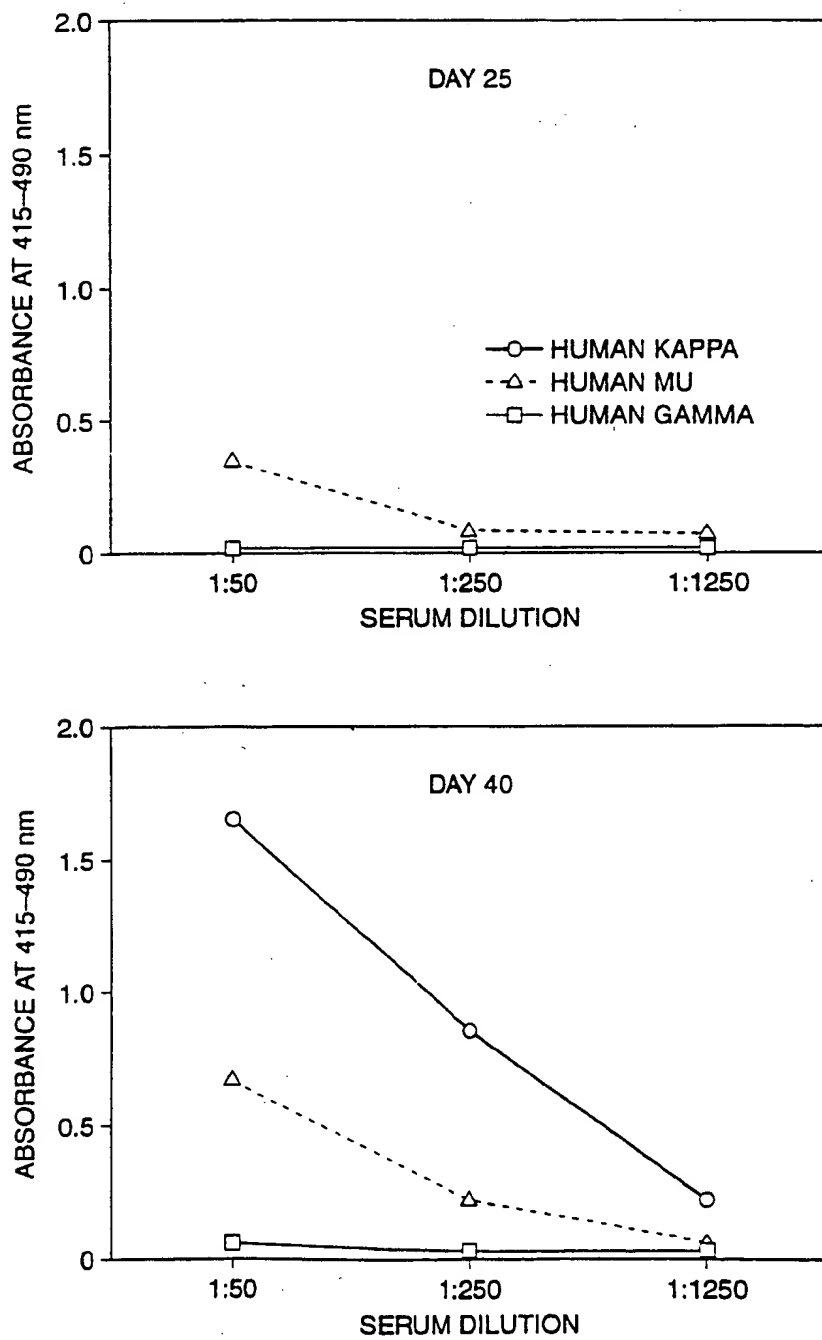
FIG. 49

FIG. 48

AAACACATTC TCTGCAGACA AATTTGAGCT ACCTTGATCT TACCTGGACA   50

GGTGGGGACA CTGAGCTGGT GCTGAGTTAC TCAGATGCGC CAGCTCTGCA   100

GCTGTGCCCA GCCTGCCCCA TCCCCTGCTC ATTTGCATGT TCCCAGAGCA   150

CAACCTCCTG CCCTGAAGCC TTATTAATAG GCTGGTCAGA CTTTGTGCAG   200

GAATCAGACC CAGTCAGGAC ACAGCATGGA CATGAGGGTC CTCGCTCAGC   250
                          MetAs pMetArgVal LeuAlaGlnL
TCCTGGGGCT CCTGCTGCTC TGTTTCCCAG gtaaggatgg agaacactag   300
euLeuGlyLe uLeuLeuLeu CysPhePro
cagtttactc agcccagggt gctcagtact gctttactat tcagggaaat   350

tctcttacaa catgattaat tgtgtggaca tttgtttta tgtttccaat   400

ctcagGTGCC AGATGTGACA TCCAGATGAC CCAGTCTCCA TCCTCACTGT   450
      GlyAla ArgCysAspI leGlnMetTh rGlnSerPro SerSerLeuS
CTGCATCTGT AGGAGACAGA GTCACCATCA CTTGTCGGGC GAGTCAGGGT   500
erAlaSerVa lGlyAspArg ValThrIleT hrCysArgAl aSerGlnGly
ATTAGCAGCT GGTTAGCCTG GTATCAGCAG AAACCAGAGA AAGCCCCTAA   550
IleSerSerT rpLeuAlaTr pTyrGlnGln LysProGluL ysAlaProLy
GTCCCTGATC TATGCTGCAT CCAGTTTGCA AAGTGGGGTC CCATCAAGGT   600
sSerLeuIle TyrAlaAlaS erSerLeuGl nSerGlyVal ProSerArgP
TCAGCGGCAG TGGATCTGGG ACAGATTTCA CTCTCACCAT CAGCAGCCTG   650
heSerGlySe rGlySerGly ThrAspPheT hrLeuThrIl eSerSerLeu
CAGCCTGAAG ATTTTGCAAC TTATTACTGC CAACAGTATA ATAGTTACCC   700
GlnProGluA spPheAlaTh rTyrTyrCys GlnGlnTyrA snSerTyrPr
ACCCACAGTG TTACACACCC AAACATAAAC CCCCAGGGAA GCAGATGTGT   750
o
GAGGCTGGGC TGCCCCAGCT GCTTCTCCTG ATGCCTCCAT CAGCTGAGAG   800

TGTTCCTCAG ATGCAGCCAC ACTCTGATGG TGTTGGTAGA TGGGGAC   847

FIG. 44

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```
CCGCCCCAGC TGCTTTGCAT GTCCCTCCCA GCCGCCCTGC AGTCCAGAGC    50

CCATATCAAT GCCTGGGTCA GAGCTCTGGA GAAGAGCTGC TCAGTTAGGA   100

ACCCCAGAGG GAACCATGGA AACCCCAGCG CAGCTTCTCT TCCTCCTGCT   150
                MetGl uThrProAla GlnLeuLeuP heLeuLeuLe
ACTCTGGCTC CCAGgtgagg ggaacatggg atggttttgc atgtcagtga   200
uLeuTrpLeu Pro
aaaccctctc aagtcctgtt acctggcaac tctgctcagt caatacaata   250

attaaagctc aatataaagc aataattctg gctcttctgg gaagacaatg   300

ggtttgattt agattacatg ggtgactttt ctgttttatt tccaatctca   350

gATACCACCG GAGAAATTGT GTTGACGCAG TCTCCAGGCA CCCTGTCTTT   400
AspThrThrG lyGluIleVa lLeuThrGln SerProGlyT hrLeuSerLe
GTCTCCAGGG GAAAGAGCCA CCCTCTCCTG CAGGGCCAGT CAGAGTGTTA   450
uSerProGly GluArgAlaT hrLeuSerCy sArgAlaSer GlnSerValS
GCAGCAGCTA CTTAGCCTGG TACCAGCAGA AACCTGGCCA GGCTCCCAGG   500
erSerSerTy rLeuAlaTrp TyrGlnGlnL ysProGlyGl nAlaProArg
CTCCTCATCT ATGGTGCATC CAGCAGGGCC ACTGGCATCC CAGACAGGTT   550
LeuLeuIleT yrGlyAlaSe rSerArgAla ThrGlyIleP roAspArgPh
CAGTGGCAGT GGGTCTGGGA CAGACTTCAC TCTCACCATC AGCAGACTGG   600
eSerGlySer GlySerGlyT hrAspPheTh rLeuThrIle SerArgLeuG
AGCCTGAAGA TTTTGCAGTG TATTACTGTC AGCAGTATGG TAGCTCACCT   650
luProGluAs pPheAlaVal TyrTyrCysG lnGlnTyrGl ySerSerPro
CCCACAGTGA TTCAGCTTGA AACAAAAACG TCTGCAAGAC CTTCATTGTT   700

TACTAGATTA TACCAGCTGC TTCCTTTACA GATAGCTGCT GCAATGACAA   750

CTCAATTTAG CATCTCTCTC TGCTTGGGCA TTTTGGGGAT CTTAAAAAAG   800

TAATCCCTTG ATATATTTTT GACTCTGATT CCTGCATTTT TCCTCAGACC   850

AAGATGGACA GCCAGGTTTA AGCACAGTTT CACAGTAATG GCCACTGGAT   900
```

FIG. 43

AGGGCGGCGC AGATGCTCAG TGCAGAGAGA AGAAACAGGT GGTCTCTGCA   50

GCTGGAAGCT CAGCTCCCAC CCCAGCTGCT TTGCATGTCC CTCCCAGCTG   100

CCCTACCTTC CAGAGCCCAT ATCAATGCCT GGGTCAGAGC TCTGGGGAGG   150

AACTGCTCAG TTAGGACCCA GACGGAACCA TGGAAGCCCC AGCGCAGCTT   200
                                 M etGluAlaPr oAlaGlnLeu
CTCTTCCTCC TGCTACTCTG GCTCACAGgt gaggggaata tgaggtgtct   250
LeuPheLeuL euLeuLeuTr pLeuThr
ttgcacatca gtgaaaactc ctgccacctc tgctcagcaa gaaatataat   300

taaaattcaa aatagatcaa caattttggc tctactcaaa gacagtgggt   350

ttgattttga ttacatgagt gcatttctgt tttatttcca atttcagATA   400
                                                   AspT
CCACCGGAGA AATTGTGTTG ACACAGTCTC CAGCCACCCT GTCTTTGTCT   450
hrThrGlyGl uIleValLeu ThrGlnSerP roAlaThrLe uSerLeuSer
CCAGGGGAAA GAGCCACCCT CTCCTGCAGG GCCAGTCAGG GTGTTAGCAG   500
ProGlyGluA rgAlaThrLe uSerCysArg AlaSerGlnG lyValSerSe
CTACTTAGCC TGGTACCAGC AGAAACCTGG CCAGGCTCCC AGGCTCCTCA   550
rTyrLeuAla TrpTyrGlnG lnLysProGl yGlnAlaPro ArgLeuLeuI
TCTATGATGC ATCCAACAGG GCCACTGGCA TCCCAGCCAG GTTCAGTGGC   600
leTyrAspAl aSerAsnArg AlaThrGlyI leProAlaAr gPheSerGly
AGTGGGCCTG GGACAGACTT CACTCTCACC ATCAGCAGCC TAGAGCCTGA   650
SerGlyProG lyThrAspPh eThrLeuThr IleSerSerL euGluProGl
AGATTTTGCA GTTTATTACT GTCAGCAGCG TAGCAACTGG CATCCCACAG   700
uAspPheAla ValTyrTyrC ysGlnGlnAr gSerAsnTrp His
TGATTCCACA TGAAACAAAA ACCCCAACAA GACCATCAGT GTTTACTAGA   750

TTATTATACC AGCTGCTTCC TTTACAGACA GCTAGTGGGG TGGCCACTCA   800

GTGTTAGCAT CTCAGCTCTA TTTGGCCATT TTGGAGTTCA AGTTGTCAAG   850

TCCAAAATTA CTTATGTTAG TCCATTGCAT CATACCATTT CAGTGTGGCT   900

FIG. 42

```
TTTTCTGGCC TGACAACCAG GGTGGCGCAG GATGCTCAGT GCAGAGAGGA    50

AGAAGCAGGT GGTCTCTGCA GCTGGAAGCT CAGCTCCCAC CCAGCTGCTT   100

TGCATGTCCC TCCCAGCTGC CCTACCTTCC AGAGCC|ATA TCAAT|GCCTG   150

TGTCAGAGCC CTGGGGAGGA ACTGCTCAGT TAGGACCCAG AGGGAACCAT   200
                                                      Me
GGAAGCCCCA GCTCAGCTTC TCTTCCTCCT GCTACTCTGG CTCCCAGgtg   250
tGluAlaPro AlaGlnLeuL euPheLeuLe uLeuLeuTrp LeuPro
agggggaacc atgaggtggt tttgcacatt agtgaaaact cttgccacct   300

ctgctcagca agaaatataa ttaaaattca aagtatatca acaattttgg   350

ctctactcaa agacagttgg tttgatcttg attacatgag tgcatttctg   400

ttttatttcc aatttcagAT ACCACCGGAG AAATTGTGTT GACACAGTCT   450
           Asp ThrThrGlyG luIleValLe uThrGlnSer
CCAGCCACCC TGTCTTTGTC TCCAGGGGAA AGAGCCACCC TCTCCTGCAG   500
ProAlaThrL euSerLeuSe rProGlyGlu ArgAlaThrL euSerCysAr
GGCCAGTCAG AGTGTTAGCA GCTACTTAGC CTGGTACCAA CAGAAACCTG   550
gAlaSerGln SerValSerS erTyrLeuAl aTrpTyrGln GlnLysProG
GCCAGGCTCC CAGGCTCCTC ATCTATGATG CATCCAACAG GGCCACTGGC   600
lyGlnAlaPr oArgLeuLeu IleTyrAspA laSerAsnAr gAlaThrGly
ATCCCAGCCA GGTTCAGTGG CAGTGGGTCT GGGACAGACT TCACTCTCAC   650
IleProAlaA rgPheSerGl ySerGlySer GlyThrAspP heThrLeuTh
CATCAGCAGC CTAGAGCCTG AAGATTTTGC AGTTTATTAC TGTCAGCAGC   700
rIleSerSer LeuGluProG luAspPheAl aValTyrTyr CysGlnGlnA
GTAGCAACTG GCCTCC|CACA GTG|ATTCCAC ATGA|ACAAA AACC|CCAACA   750
rgSerAsnTr pPro
AGACCATCAG TGTTTACTAG ATTATTATAC CAGCTGCTTC CTTTACAGAC   800

AGCTAGTGGG GT                                            812
```
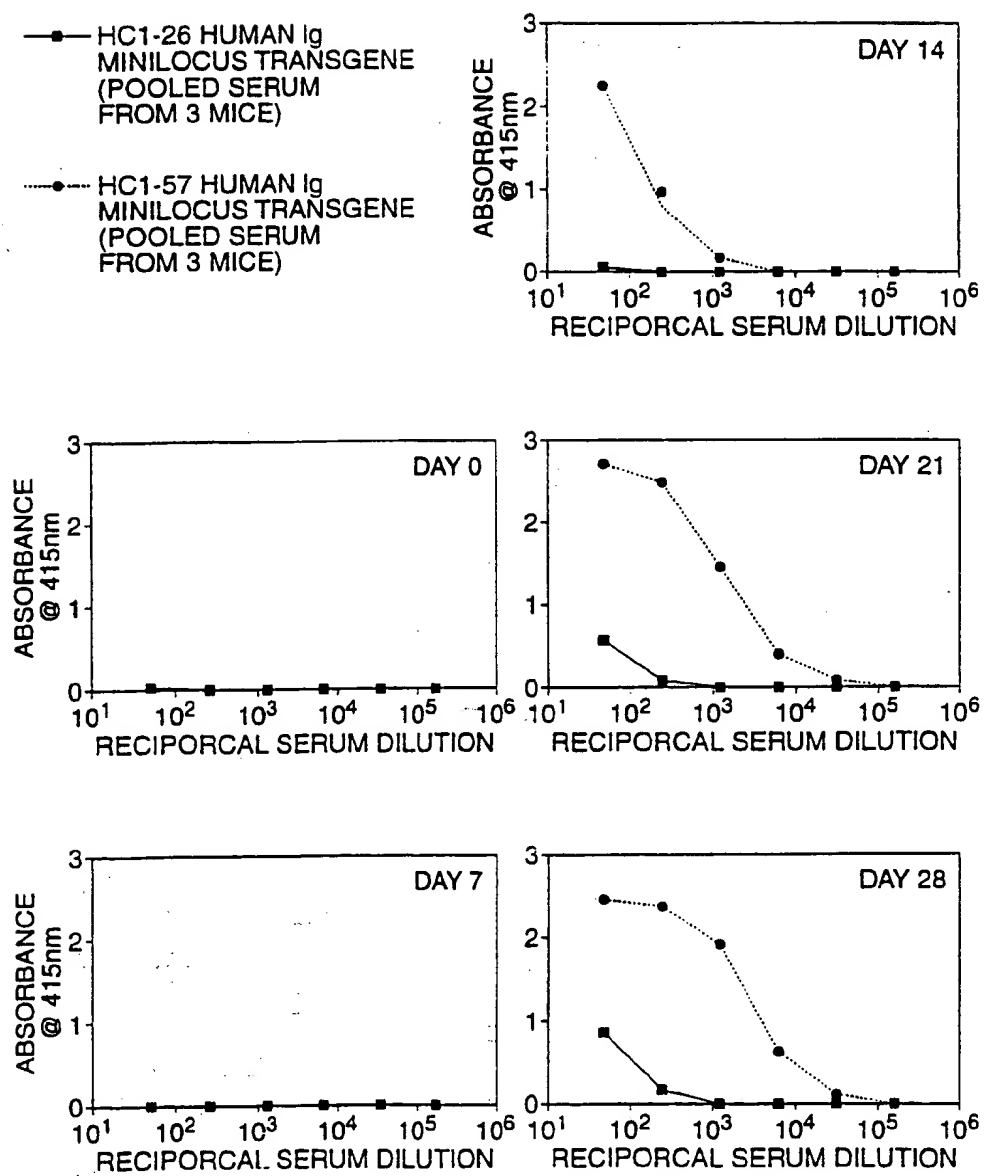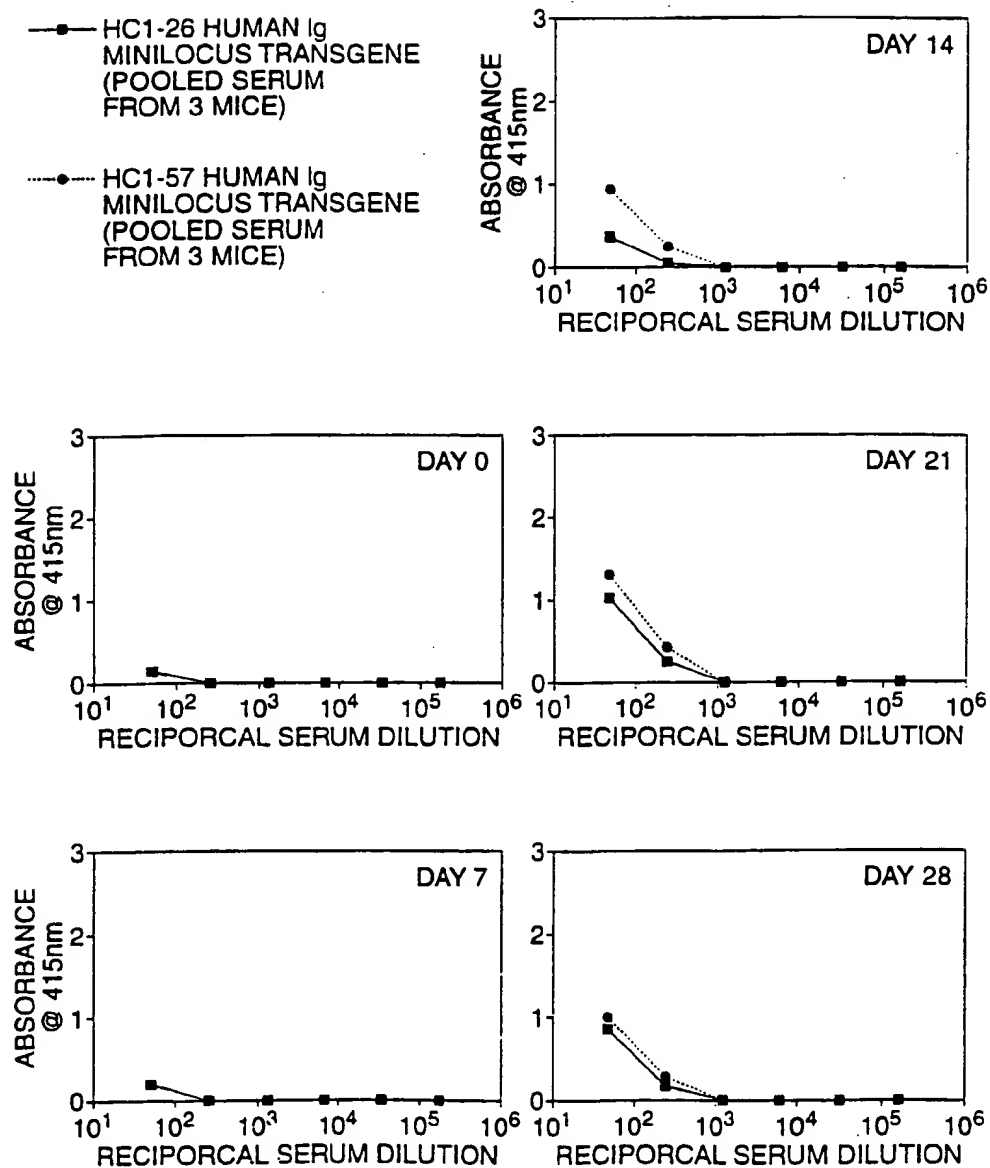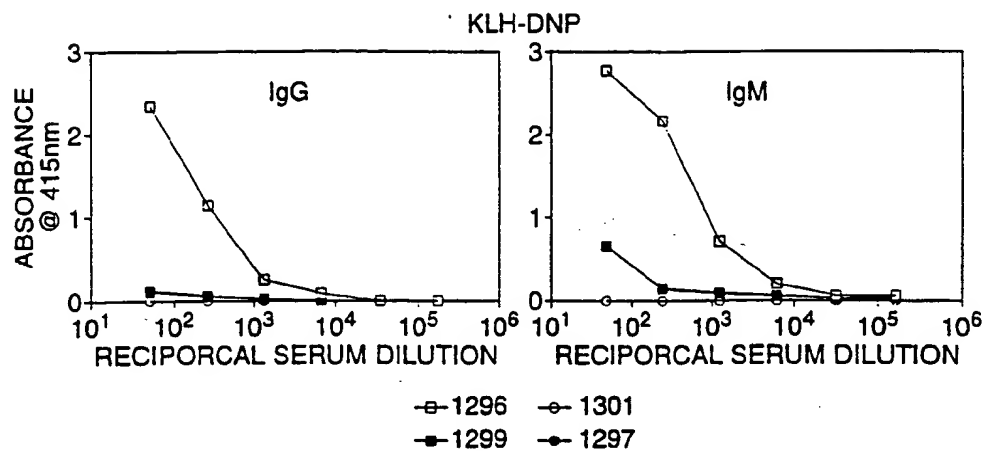
FIG. 41

FIG. 40

FIG. 39

FIG. 38

FIG. 37A

FIG. 37B

FIG. 37C